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A new β-glucosidase, "W_1", was demonstrated by electrophoretic analysis to be in conidial washes of several exotic strains of Neurospora crassa. The biochemical and genetic characteristics of "W_1" were compared with those of established aryl β-glucosidase and cellobiase in order to study diversity in a species.

Aryl β-glucosidase and "W_1" in the conidial wash of exotic strain of P-212 were separated by three methods: electrophoresis on cellulose polyacetate, where "W_1" was less mobile; Bio-Gel P-200 column fractionation, where "W_1" (the smaller enzyme) was eluted last; and calcium phosphate gel adsorption, where aryl β-glucosidase was adsorbed, and "W_1" was in the supernatant fraction.

The physical properties of "W_1" were determined using a purified preparation of "W_1" which was obtained after treatment of a conidial wash with calcium phosphate gel. The new enzyme was relatively thermostable at 60 C, with a half-life of 16 - 17 minutes. The enzyme had a pH optimum of 5.0 in potassium phosphate and in citrate phosphate buffer. A Michaelis constant of 1.98×10^{-5} Molar p-nitrophenyl β-D glucose was determined for Neurospora "W_1".

Although "W_1" was found in conidial washes of strains only 72 hours old (the first day of conidiation), attempts to induce the production of "W_1" in mycelia of any age were unsuccessful.

The genetic studies showed that "W_1" was passed to succeeding generations in crosses with wild-type strains. The new β-glucosidase and aryl β-glucosidase, but not cellobiase, appeared to be coordinately regulated by the gluc locus.

Electrophoretic analysis revealed a second new β-glucosidase, "W_2", in conidial washes of many exotic and wild-type strains. Electrophoretically, "W_2" was always closely associated with aryl β-glucosidase, but attempts to separate the two enzymes by other methods were unsuccessful.

From these studies, it is apparent that Neurospora produces at least three extracellular β-glucosidases. All three seem to be regulated by the gluc locus, and at least two of them (aryl β-glucosidase and "W_1") have similar kinetic properties.

IDENTIFICATION OF TWO NEW β-GLUCOSIDASES OF *NEUROSPORA CRASSA*

by

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APPROVAL SHEET

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INTRODUCTION

Fungi have the advantage of being studied as microorganisms (due to their characteristic short life cycle, simplified mating characteristics and availability for dependable genetic analysis), and yet, they may have the same protein regulation as higher organisms (due to their eucaryotic chromosomes). In a recent review by Gross (1969), it was stated that while most of the multicistronic systems in fungi are non-randomly dispersed, several functionally related genes are tightly linked.

For the past ten years, research has been done on the β-glucosidase system of Neurospora crassa. In a finding which paralleled Zalokar and Cochrane's (1956) observation of diphosphopyridine nucleotidase associated with conidia and sporulation of Neurospora, Berger and Eberhart (1961) reported finding p-nitrophenyl β-D-glucosidase activity and cellobiase-transglycosidase activity in conidial washes. Later, Eberhart (1962) reported a mutant strain, gluc-1, with less than 10% of the normal aryl β-glucosidase activity but able to grow at wild-type levels when induced with cellobiose or carboxymethyl cellulose. The mutation was classified as a dominant regulatory gene which reduced the level of aryl β-glucosidase activity but had no effect on cellobiase production (Mahadevan and Eberhart, 1962; Eberhart, Cross, and Chase, 1964). Eberhart, et al. (1964) reported that Neurospora also produced at least two cellulases, neither affected by the gluc-1 gene. Although aryl β-glucosidase and cellobiase have been separated and characterized, no dissociable inhibitor (gluc-1 product) such as the lac (Gilbert and Müller-Hill, 1966,

1967) or the lambda phage (Ptashne, 1967a,b) repressor proteins has been isolated (Mahadevan and Eberhart, 1964a,b).

Theoretically, the β-glucosidases have a role in the carbon cycle by participating in the degradation of cellulose to CO_2 and water, but it appears that they are not coordinately regulated. Cell-1, a recessive regulatory gene (Meyers and Eberhart, 1966), leads to the constitutive production of cellulase and cellobiase, which have been postulated to participate in cellulose degradation as follows (Eberhart, 1968):

$$\text{native cellulose} \xrightarrow[\text{cellulase}]{\text{unidentified}} \text{modified cellulose} \xrightarrow{\text{cellulase}}$$

$$\text{cellobiose} \xrightarrow{\text{cellobiase}} \text{glucose}$$

Aryl β-glucosidase, primarily a mural enzyme, is regulated by the gluc locus, where the gluc-1 allele permits less than 10% of the normal production of the enzyme, and a gluc-2 allele results in less than 1% of normal activity (Eberhart and Beck, 1970). Aryl β-glucosidase and cellobiase hydrolyze aryl β-glucosides into an aglycone and glucose (Eberhart, 1968).

Although two regulatory genes have been found for the β-glucosidase system, the nature of the structural genes of these β-glucosidases is not known. The following questions remain to be answered: 1) Are the structural genes clustered in Neurospora as they are in the lac operon of E. coli? 2) Are the β-glucosidase enzymes produced directly (i.e., according to the one gene-one enzyme hypothesis or to the one gene-one polypeptide hypothesis)?

The purpose of this study was to examine a number of exotic (foreign) strains of N. crassa for biochemical diversity in their exogenous

β-glucosidase (aryl β-glucosidase). Mutant structural genes, responsible for natural diversity, could then be mapped (along with the regulatory genes), and the results could be analyzed and compared with the operon concept proposed by Jacob and Monod (1961).

An initial survey of the exotic strains revealed a new enzyme (designated "W_1"), in addition to previously reported aryl β-glucosidase, in several of the strains. The new enzyme seemed significant as to its role in the β-glucosidase system of Neurospora and its contribution to the diversity of a species. Since "W_1" was a new phenomenon (rather than a simple variation of a previously characterized enzyme), its biochemical and genetic characteristics had to be determined in reference to the β-glucosidase system previously described in an attempt to answer the following questions:

1. Under what conditions, and in which strains, is "W_1" produced?

2. Is the structure of "W_1" directly determined by information from a unique structural gene; or is it directly related to one of the previously reported β-glucosidases, differing only in its electrophoretic mobility (due to action of a protease or to its association with a carbohydrate or other cellular component)?

3. Is "W_1" regulated by the cell locus, the gluc locus, or by some, as yet, undefined locus?

4. How do the physical properties of "W_1" compare with those of aryl β-glucosidase and cellobiase?

5. Can "W_1" be simultaneously induced with aryl β-glucosidase and cellobiase?

As the study progressed, electrophoresis of conidial washes revealed another exogenous β-glucosidase (designated "W_2") in most exotic and wild-type strains. While the same questions were asked of "W_2" (as of "W_1"), they were answered with much less success.

MATERIALS AND METHODS

Chemicals

N-acetyl-glucosamine, p-nitrophenyl-β-D-glucopyranoside (PNPG), ortho-nitrophenyl-β-D-galactopyranoside (ONPG), and maltose were obtained from Calbiochem. The Glucostat Special reagent was purchased from Worthington Biochemical Corporation. Gentiobiose, trehalose, and Tris-hydroxymethylaminomethane (Tris) were products of Sigma Chemical Company. Lactose, glucose, and glycerol were obtained from Matheson Coleman & Bell. Salicin was a product of Nutritional Biochemicals Company. D-galactose and Bacto-casitone were purchased from Difco. 4-methyl-umbelliferyl-β-D-glucopyranoside (umbelliferone) was obtained from Mann Research Lab. Bio-Gel P-200 was purchased from Bio-Rad Laboratories, and Sephadex G-200 was from Pharmacia, Inc. Carbowax - Polyethylene Glycol Compound 20-M was from Union Carbide. Lyphogel and all electrophoretic materials were products of Gelman. Isopropyl-β-D-thiogalactopyranoside (thiogalactoside) was obtained from N.K. Ritchmyer, National Institute of Health.

Selection of Strains

This study was begun by surveying a variety of exotic strains (obtained from the Fungal Genetics Stock Center (F.G.S.C.) and from Dr. David Perkins of Stanford University) for qualitative and quantitative differences in exogenous β-glucosidase. (The strains, their origins, and their electrophoretic patterns are summarized in Table 1). Electrophoresis (on cellulose polyacetate) of crude enzyme preparations (conidial washes) from wild type strains had demonstrated one band of β-glucosidic activity

TABLE I

ORIGINAL OBSERVATIONS OF EXOTIC STRAINS

STRAIN (FGS)	SOURCE	ORIGIN	β-GLUCOSIDASES
3	D.D. Perkins	Japan	Y
30	D.D. Perkins	Philippines	Y
56	D.D. Perkins	Taiwan	Y
57	D.D. Perkins	Taiwan	Y
58	D.D. Perkins	Taiwan	Y
61	D.D. Perkins	Taiwan	Y
64	D.D. Perkins	New Guinea	W_1 + Y
91	D.D. Perkins	Australia	W_1 + Y
113	D.D. Perkins	Australia	W_1 + Y
142	D.D. Perkins	Indonesia	W_1 + Y
153	D.D. Perkins	Indonesia	Y
168	D.D. Perkins	Indonesia	Y
190	D.D. Perkins	Indonesia	Y
204	D.D. Perkins	Indonesia	Y
212	D.D. Perkins	Indonesia	W_1 + Y
249	D.D. Perkins	Malaya	Y
266	D.D. Perkins	Malaya	Y
271	D.D. Perkins	Malaya	W_1 + Y
278	D.D. Perkins	Singapore	Y
285	D.D. Perkins	India	Y
291	D.D. Perkins	India	Y
296	D.D. Perkins	India	Y
321	D.D. Perkins	India	Y
330	D.D. Perkins	India	Y
343	D.D. Perkins	India	Y
348	D.D. Perkins	India	Y
349	D.D. Perkins	India	Y
429	FGSC	Puerto Rico	Y
430	FGSC	North Africa	Y
431	FGSC	Java	Y
432	FGSC	Fiji	Y
433	FGSC	Philippines	Y
434	FGSC	Liberia	Y
435	FGSC	Fiji	Y
436	FGSC	Singapore	Y
643	FGSC	New Zealand	Y
847	FGSC	Lein	Y
851	FGSC	Costa Rica	Y
852	FGSC	Costa Rica	W_1 + Y
961	FGSC	Liberia	Y
967	FGSC	Liberia	Y

(using umbelliferone as the substrate), designated aryl β-glucosidase or "Y". Of the 41 exotic strains electrophoresed, seven of them (P-64, P-91, P-113, P-142, P-212, P-271, and FGSC-852) demonstrated two bands, rather than the one band (aryl β-glucosidase) previously reported. P-212 was selected for more intensive study, as it had two strong (enzymatically active) bands while the other strains seemed to have one strong band (aryl β-glucosidase) and one weak band of "W_1" activity.

The following wild-type strains were often used as controls and in genetic crosses: 74-OR8-1a, 74-OR23-1A, STA-4, E-692a, and FGSC-89601. A gluc-2 strain (less than 1% production of aryl β-glucosidase activity), RB-1(83)a, was used in crosses to determine the regulation of "W_1" found in the conidial wash.

Growth of Cultures for Crude Conidial Wash Preparation

The strains were maintained for transfer at 25 C on 5 ml of modified glycerol complete medium (Eberhart, et al., 1964), containing 0.1% Bacto-casitone, 0.25% yeast extract, 1.5% agar, 1% Vogel's minimal salts (Vogel, 1956), 1% vitamin stock solution, and 0.8% glycerol. To insure culture continuity, wild-type strains and P-212 were occasionally transferred from silica gel.

Based on the solubility of β-glucosidases in water (Eberhart, 1961), crude enzyme preparations were made by adding glass distilled water to conidiated cultures grown in Erlenmeyer flasks of varying sizes, depending on the amount of enzyme preparation needed. Table 2 shows the relationship of flask size, amount of medium, and quantities of water necessary for a conidial harvest.

At the beginning of this study conidial washes were harvested by a method modified from Eberhart (1961). Conidial suspensions were made by adding glass distilled water to seven day cultures, which were fully conidiated at that time (Table 2). With the cotton plug in place, each flask was shaken to suspend the conidia. The conidial suspension was filtered through glass wool to remove any mycelium, and the filtrate was centrifuged at 5 C in an automatic refrigerated centrifuge (Sorvall Super-speed RC2-B) at a relative centrifugal force of 17,300 X g (RCF maximum) for 20 minutes. The supernatant fraction was put into dialysis tubing (Fisher Scientific Company) and concentrated with a polyethylene glycol compound (Carbowax) in the refrigerator (several hours or overnight) until the desired concentration was reached (usually five to ten-fold). The concentrate was then frozen, without loss of activity, for future assay or for purification.

TABLE 2

CULTURE GROWTH AND CONIDIAL HARVEST

Flask (ml)	Medium (ml)	Water (ml) Long Method	Water (ml) Short Method
2000	200	150-200	35
500	50	50-75	20
250	25	-	15
125	15	-	10
tubes	5	-	2

A simplified method, which was satisfactory for routine genetic tests involving electrophoresis, was developed to eliminate the necessity

of concentrating the conidial wash. A limited amount of glass distilled water (Table 2) was added to tubes or flasks of conidiated cultures to make concentrated suspensions of conidia, which were filtered through glass wool and centrifuged as above. The supernatant fraction could then be used directly or frozen for future study.

Although routine procedures required very clean conditions only during a conidial wash harvest, conidial suspensions prepared for induction studies had to be aseptic.

Mycelial Growth and Induction

Equal amounts of sterile conidial suspension were introduced into 250 ml Erlenmeyer flasks, containing 80 ml of Vogel's single-strength medium (Vogel, 1964) with vitamins and 2% sucrose as the carbon source. The mycelia were grown for various lengths of time at 25 C in an incubator shaker (Gyrotory Shaker model G-25, manufactured by New Brunswick Scientific Company), or in a reciprocating incubator shaker bath (model RW-650, manufactured by New Brunswick Scientific Company).

After a specified growth period (minimum of 12 hours), the mycelia were aseptically filtered on Whatman No. 1 filter paper, washed with sterile water several times, and transferred to similar flasks containing 80 ml of sterile 0.1 M potassium phosphate buffer (pH 5.0) plus inducer (Cellobiose is the most common inducer of β-glucosidases in Neurospora) to give a final concentration of 1.0 millimolar; but in some instances, other specified compounds were used in an attempt to induce the early production of "W_1". After six hours of induction, the mycelial pads were harvested by filtration on Whatman No. 1 filter paper and frozen at -25 C for future extraction of mycelial protein. The supernatant

fraction (the inducing buffer) was concentrated with Carbowax and frozen for future examination for the production of exogenous β-glucosidase.

Crude mycelial extracts were prepared by a method modified from Horowitz and Shen (1952). Frozen mycelial pads were broken into pieces and ground in an Omni-Mixer chamber (model No. 115, manufactured by Ivan Sorvall, Inc.) with 0.01 M potassium phosphate buffer (pH 5.0) and glass homogenizing beads (No. 16-220, VirTris Company) as summarized in Table 3.

TABLE 3

PROTEIN EXTRACTION FROM MYCELIA

Crude Extract	Mycelial Wet Weight (g)	Glass Beads (g)	Buffer (ml)	Extration (min)	Setting
Micro dilute	0.2	1.0	3.0	10	2.0
Micro concentrated	1.2	1.0	3.0	20	2.0
Macro dilute	1.0	5.0	15	10	4.5
Macro concentrated	8.0	7.0	20	20	4.5

The homogenates were allowed to extract for one hour at 5 C with frequent stirring, after which they were centrifuged in the Sorvall refrigerated centrifuge at 17,300 X g for 20 minutes. The supernatant portions were then frozen for future study.

Induction and Extraction of Conidia and Six Hour Cells

The cells were induced and extracted according to a method modified from Eberhart and Beck (1970). This procedure was used in an experiment to determine if, and when, "W_1" was inducible (by cellobiose) during the life cycle of Neurospora. In order to keep the culture conditions as uniform as possible, the conidia and six hour cells were not treated with

acid (which would have destroyed the patent β-glucosidase activity) prior to induction. An aseptic conidial suspension of P-212 was obtained and centrifuged for 10 minutes at 3,000 X g in a Sorvall type SP/X centrifuge (Ivan Sorvall, Inc.). After two washings with sterile distilled water, the cells were suspended in 0.1 M potassium phosphate buffer (pH 5.0). The cells were then inoculated (into 250 ml Erlenmeyer flasks containing 80 ml of the same buffer, plus 0.1 mM of cellobiose) with an inoculum that had an optical density reading in the final suspension of 0.050 to 0.100 at 600 nm (equivalent to 3×10^6 to 6×10^6 cells per ml of induction medium) in the Beckman-Spinco Spectrophotometer (model No. 151, Beckman Instruments).

The inoculated flasks were incubated at 25 C in an incubator shaker (Gyrotory Shaker model No. G-25, New Brunswick Scientific Company) for six hours. The contents were centrifuged at 12,100 X g for 20 minutes at 5 C. The supernatant fraction was then concentrated with Carbowax and frozen for future assay. The pellet of cells was suspended in 5 ml of 0.01 M potassium phosphate buffer (pH 5.0) with 2.0 g of chilled glass homogenizing beads. The sample was placed in an ice-water-salt bath until its temperature was 0 C. The suspension was disrupted in a Sonifier (model S-110, Branson Instruments) at a setting of 4.3 amps. Six 15 second bursts were applied, interspersed with cooling periods (in the bath) for the temperature to reach 5 C. The cooling periods lessened enzyme inactivation due to heat from the sonifier.

The disrupted cells were placed in an ice bath (to obtain maximum extraction) and frequently agitated for one hour. The samples were centrifuged at 17,300 X g for one hour. The supernatant fractions were frozen for future assay.

Growth of Strains for Genetic Analysis

Steep slants were made in test tubes containing 5 ml of corn meal agar, which was composed of 1.7% corn meal agar and 10% vitamin stock solution. Protoperithecia from the maternal parent were ripe and ready for inoculation with a strain of the opposite mating type (paternal parent) seven to ten days after the initial inoculation. After a week or ten days, the perithecia discharged their spores, and the spores could be isolated at that time. The spores for these studies were usually isolated after they had had time to ripen and darken, after two weeks or more.

The spores were isolated in the following manner: A drop of sterile distilled water was added to an area in the crossing tube dense with spores. The water, containing suspended spores, was transferred with a sterile loop to a 6% agar block treated with sodium hypochlorite, 5.25% (Clorox), which killed the mycelia and prevented carry-over contamination from the parental strain. Spores, picked one by one, were transferred to very small test tubes, containing 1 ml of modified glycerol complete medium. After the desired number of spores had been isolated, they were immediately heat-shocked at 60 C for 20 minutes and then incubated at 25 C.

In genetic studies designed to determine the regulation of "W_1", RB-1(83)a (a gluc-2 strain) was crossed with P-212. β-glucosidaseless mutants were screened using the esculin-iron test described by Eberhart, et al. (1964). Strains with a mutant phenotype were unable to hydrolyze the esculin quickly, and the medium remained comparatively unchanged after 24 hours. Strains with a wild-type phenotype had active β-glucosidase which attacked the esculin, producing esculetin and glucose. The

esculetin, reacting with iron in the medium, caused a darkening of the medium proportional to the amount of β-glucosidase activity. Strains able to produce β-glucosidase were transferred to flasks containing modified glycerol complete medium, incubated, harvested, and electrophoresed in the standard way.

Protein Determination

The protein in a sample was determined by the method of Lowry, et al. (1951) using varying concentrations of human serum albumin as reference standards.

Electrophoresis

When this study was begun, electrophoresis was done in a Gelman Electrophoresis Chamber using a cellulose polyacetate medium. Ten microliter samples were pipetted onto strips of Sepraphore III (1" X 6 3/4" or 1" x 6" strips of cellulose polyacetate, manufactured by Gelman Instrument Company), which had been presoaked in chilled 0.1 M potassium phosphate buffer at pH 6.0. Two different samples could be pipetted onto each strip (one at the top and one at the bottom of each strip), allowing 14 samples to be run in the chamber, which contained 450 ml of chilled 0.1 M potassium phosphate buffer at pH 6.0 (Electrophoresis was originally done at pH 6.0 since the pH optimum of cellobiase was 6.1 and that of aryl β-glucosidase was 5.0 to 5.2 (Eberhart and Beck, 1970). Later, electrophoresis was done at pH 5.0 and at pH 7.0, but "W_2" was often indistinguishable at these pH conditions, and the separations did not appear to be as complete.). The origins (2.25" from the end of the strip) were carefully marked and aligned, and the strips were tensioned with micro-glass slides. The apparatus was put into the refrigerator and

was attached to a voltage regulated power supply set at 250 V. Enzymes migrated towards the cathode for one hour (the time was later reduced when it was discovered that a successful separation could be accomplished in 30 minutes). After a separation, the cellulose polyacetate strips were placed on filter paper strips pre-soaked with a saturated substrate solution and covered with plastic wrap to prevent evaporation and facilitate recording of the data.

For routine studies, and unless otherwise noted, 4-methyl-umbelliferyl-β-D-glucopyranoside (umbelliferone) was the substrate. As the β-glucosidic bonds were cleaved, a fluorescence could be observed using an ultraviolet light. The bands were marked as they occurred, and later transferred to a permanent record.

Some of the less frequently used substrates required different techniques in order to observe enzyme activity. When the β-glucoside esculin (a fluorescent substance) was the substrate, bands of quenched fluorescence were observed with an ultraviolet light and scored as evidence of β-glucosidase activity. When PNPG was the substrate, the cellulose polyacetate strips were placed on filter paper strips soaked in a 10% saturated solution of PNPG. As the β-glucosidic bonds were cleaved, a chromogenic substance was released. After 10 minutes the reaction was stopped, and the yellow color was intensified for scoring by placing filter paper strips soaked with 1 M Tris on top of the electrophoresis strips.

The non-chromogenic carbohydrate substrates were used in association with Glucostat Special reagent. The electrophoresis strips were placed on top of filter paper strips pre-soaked in saturated solutions of cellobiose, trehalose, maltose, sucrose, and lactose, and covered with plastic

wrap to prevent evaporation. After 15 minutes, filter paper strips, which had been soaked in a Glucostat Special solution (the chromagen, dissolved in 2 ml of glass distilled water, was added to the Glucostat Special, which had been dissolved in 0.2 M potassium phosphate buffer at pH 7.4) were placed on top of the cellulose polyacetate strips. In the presence of glucose (a cleavage product of the disaccharides), the coupled reaction resulted in a red color which could be scored.

Later in the study, a shorter method of electrophoresis was developed using the Gelman Sepratek System. A pre-soaked (in 0.1 M potassium phosphate buffer, pH 6.0) strip of Sepraphore III Sepratek Support Medium was blotted, tensioned on the support bridge, and placed in the buffer chamber (model No. 51156, Gelman Instrument Company), which contained 200 ml of chilled buffer. Fresh or thawed enzyme samples were pipetted into the sample wells of the Sepratek System. After the applicator was loaded and the samples applied to the strip, the origin was marked. The apparatus was placed in the refrigerator with the voltage regulator set at 200 V for 20 minutes. After the run, the cellulose polyacetate strip was placed on a piece of filter paper pre-soaked in a solution of saturated umbelliferone. The strip was mounted on a sheet of plastic and covered with plastic wrap to allow the bands to be marked when the fluorescence appeared.

The Sepratek method had the advantages of taking less time for a run and of producing consistent data (since samples were applied simultaneously, in equal amounts, and at the same origin). However, separations were limited to eight samples, and it was difficult to obtain an accurate reading with a dilute sample. With a dilute sample, 30-60 minutes were

sometimes required to analyze the bands. Often by the time activity was visible, the band was no longer distinct due to diffusion effects. This problem could be alleviated by either concentrating the preparation or by making several applications of enzyme. The original method had the advantages of permitting more samples to be scored at one time (in a longer run), but the arbitrary alignment of sample origins, as well as the time elapsed between the application of the first enzyme and the last, made repeatability more difficult.

Ammonium Sulfate Precipitation

It had been previously reported that cellobiase and aryl β-glucosidase from mycelial extracts of Neurospora could be separated by treating the crude preparation with different concentrations of ammonium sulfate (Eberhart, et al., 1964). According to Colowick and Kaplan (1955), enough ammonium sulfate was added to a crude conidial wash from P-212 to make a 40% saturated solution. The mixture was stirred, allowed to equilibrate at 4 C for one hour, and centrifuged at 12,100 X g for 30 minutes at 5 C. The precipitate was suspended in potassium phosphate buffer (pH 6.0) and dialyzed exhaustively against distilled water in the refrigerator. Ammonium sulfate was then added to the supernatant fraction to give a 95% saturation. The precipitate was treated as above. After electrophoresis revealed that the majority of the β-glucosidase activity was in the 40 - 95% fraction, the suspension was salted to give the following degrees of saturation: 0 - 60%, 60 - 70%, 70 - 80% saturation, and 90 - 95%. After each salting, the sample was equilibrated, centrifuged, and dialyzed as above. The fractions were then electrophoresed to determine if a separation of aryl β-glucosidase and "W_1" had occurred.

Calcium Phosphate Gel Adsorption

Calcium phosphate gel was made according to Keilin and Hartree (1938). A conidial wash preparation from P-212 was concentrated and dialyzed exhaustively against distilled water. The crude preparation was put into polycarbonate centrifuge tubes with equal amounts of calcium phosphate gel. Citrate phosphate buffers (0.1 M) of pH 4.1, 4.6, 5.2, 5.8, 6.5, and 7.0 were added to each tube to give a final molarity of 0.01 M. The samples were placed in an ice bath for 30 minutes and periodically agitated. After centrifugation at 12,100 X g for 10 minutes at 5 C, the supernatant fractions were evaluated electrophoretically.

The two enzymes were not separated by differential adsorption onto calcium phosphate gel using citrate phosphate buffers; therefore the procedure was repeated with potassium phosphate buffer. The crude enzyme preparation was mixed with an equal amount of calcium phosphate gel. 0.1 M potassium phosphate buffer (pH 5.0) was added to give a final molarity of 0.01 M. The supernatant fraction was electrophoresed following centrifugation. The "partially purified 'W_1' preparation" referred to in this thesis was obtained by this method.

Gel Filtration

Gel filtration was used to separate the exogenous β-glucosidases of strain P-212 and to determine the size of "W_1" relative to that of aryl β-glucosidase. One ml of a dialyzed, concentrated, conidial wash preparation of P-212 was pipetted onto a column (packed in a Sephadex Column, K 15/30, manufactured by Pharmacia) of Sephadex G-200 and later onto a column of Bio-Gel P-200. The eluant in both cases was 0.1 M potassium phosphate buffer, pH 6.0. One ml samples were collected, assayed with PNPG, and scored electrophoretically.

Assay Methods

P-nitrophenyl β-D-glucoside (PNPG) was the substrate used in routine quantitative assays of β-glucosidase activity. This assay is based on the release of a chromogenic p-nitrophenyl moiety (at an alkaline pH) when the β-glucosidic link of PNPG is hydrolyzed. The discontinuous colorimetric method described by Eberhart (1961) was slightly modified for this study. Stock solutions of PNPG (10 mg/ml) was kept frozen for use. 0.8 ml of PNPG was added to 0.2 ml of the sample to be assayed to give a final substrate concentration of 1 mg/ml. After 10 minutes incubation at 25 C, the reaction was stopped with 0.5 ml of 1 M Tris, resulting in an alkaline pH of 9.2 which converted all of the released p-nitrophenol to its colored form. Controls, in which enzyme samples were mixed with the substrate after the addition of the Tris, were run on each sample. The optical density of each sample was read at 410 nm on the Spectrocolorimeter, and the readings were recorded as optical density/minute. The readings were then converted to international enzyme units (one unit is defined as one mole of PNPG released per minute at 25 C) based on a standard curve (Beck, 1969).

An attempt was made to design an assay using cellobiose as the substrate. The cellobiose was used in conjunction with Glucostat Special, a coupled enzyme system (with a minimal trace of carbohydrases) which would produce an oxidized chromogen in the presence of glucose. The Glucostat Special was made in 20 ml of 0.2 M potassium phosphate buffer, pH 7.0 (the pH optimum of Glucostat). A 0.1 M solution of cellobiose was made with 0.01 M potassium phosphate buffer, pH 5.0 (pH optimum of "W_1"). At timed intervals, 0.5 ml of cellobiose was added to tubes

containing 0.2 ml of enzyme sample. At 0, 10, 20, and 30 minutes, 0.3 ml of Glucostat was added. Each reaction was stopped after 10 minutes by adding 0.1 ml of 4 N HCl to each tube. Controls were made by incubating cellobiose with Glucostat (without enzyme) for 10 minutes, and by adding enzyme samples to tubes containing acid, cellobiose, and Glucostat. The samples were then read at 400 nm on the spectrophotometer.

RESULTS

Discovery of a New β-Glucosidase

This study was originally designed to examine the diversity of aryl β-glucosidase in exotic strains of Neurospora. When electrophoresis of the conidial wash of P-212 demonstrated two bands of β-glucosidase activity (using umbelliferone as the substrate) the emphasis was shifted to the purification and characterization of the new enzyme. Past investigators (Eberhart, 1961; Eberhart, et al., 1964; and Eberhart and Beck, 1970) have only reported aryl β-glucosidase activity in conidial washes. The exogenous enzyme, named aryl β-glucosidase (designated as "Y" for the remainder of this paper) because of its strong affinity for PNPG, had a relatively consistent electrophoretic mobility (in all strains examined) in 0.1 M potassium phosphate buffer at pH 6.0. The new enzyme (designated "W_1") had a relatively consistent electrophoretic mobility in seven of the 41 exotic strains. The characteristic electrophoretic mobilities of individual β-glucosidases of Neurospora are shown in Figure 1a. Typical electrophoretic patterns which have been observed in Neurospora are shown in Figure 1b (patterns with the Sepratek method are illustrated in Figure 11). The "typical" electrophoretic pattern of β-glucosidases from the conidial wash of wild-type strains and of most exotic strains is denoted Class 1 in the Figures. The pattern found in P-212, P-64, P-113, P-142, P-271, and FGSC-852 is denoted Class 3.

Preliminary data gathered on P-212 indicated that "W_1" was able to hydrolyze the β-glucosidic bonds in umbelliferone, PNPG, and esculin,

Figure 1a. Characteristic Electrophoretic Mobilities of Neurospora β-Glucosidases.

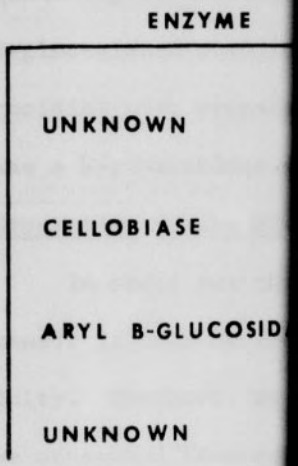
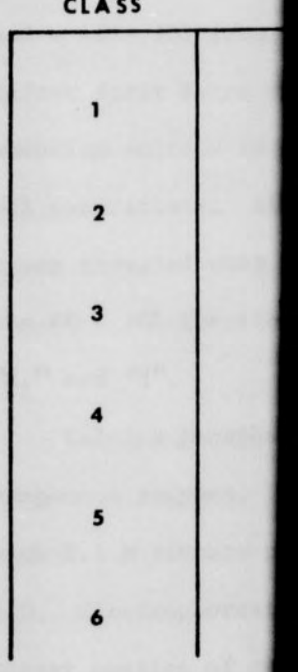
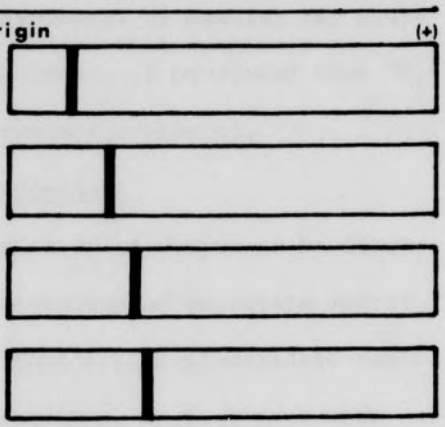
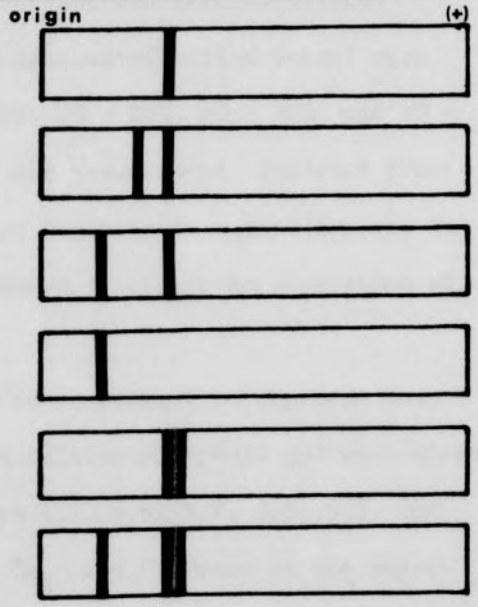


Figure 1b. Typical Electrophoretic Patterns of the β-Glucosidases in Neurospora (analogous to Figure 11)

Class	Origin
1	Conidial wash
2	Mycelial extract
3	Conidial wash
4	Conidial wash supernate after treatment with calcium phosphate gel
5	Conidial wash
6	Conidial wash



ENZYME	DESIGNATION	ELECTROPHORESIS ON CELLULOSE POLYACETATE
UNKNOWN	W_1	
CELLOBIASE	X	
ARYL B-GLUCOSIDASE	Y	
UNKNOWN	W_2	

CLASS	CHARACTERISTIC ENZYMES	ELECTROPHORESIS ON CELLULOSE POLYACETATE
1	Y	
2	X + Y	
3	W_1 + Y	
4	W_1	
5	Y + W_2	
6	W_1 + Y + W_2	

with the hydrolysis products (observed as fluorescence, yellow color, and quenching of fluorescence, respectively) increasing over a period of time. β-glucosidase activity ("W_1" and "Y") was destroyed by heating the crude conidial wash preparation at 100 C for one minute. I concluded that "W_1" was a β-glucosidase (and not due to a fluorescent artifact).

Separation of the New Enzyme and Aryl β-Glucosidase

In order for the physical characteristics of an enzyme to be determined, it must be free of any enzymes with overlapping substrate specificity. Eberhart, et al. (1964) have shown that aryl β-glucosidase could be separated from cellobiase (in mycelial extracts) by treatment with differing saturations of ammonium sulfate. They found "Y" to be predominantly in the 65 - 70% fraction. In this study, a crude conidial wash of P-212 was treated with ammonium sulfate to give a 0 to 40% saturation and a 40 - 95% fraction. The 40 - 95% fraction was further treated (after first being exhaustively dialyzed against distilled water) with ammonium sulfate to give 0 - 60%, 60 - 70%, 70 - 80%, 80 - 90%, and 90 - 95% saturations. Although PNPG assay of the resuspended, dialyzed fractions revealed that the greatest amount of β-glucosidase activity was in the 60 - 70% fraction, electrophoresis showed little or no separation of "W_1" and "Y".

Calcium phosphate gel was then used in an attempt to separate the exogenous enzymes. The conidial wash and calcium phosphate gel were mixed with 0.1 M citrate phosphate buffers of pH 4.1, 4.6, 5.2, 5.8, 6.5, and 7.0. Electrophoresis revealed that both "W_1" and "Y" were in the supernatant portion of every mixture following treatment. I concluded that there may have been some interaction between the gel and the citrate

which prevented a differential adsorption of "W_1" and "Y".

The procedure was then repeated using 0.1 M potassium phosphate buffer at pH 5.0 (a median range pH value). After the mixture had had time to react, it was centrifuged. When the supernatant portion was electrophoresed, only "W_1" was strong enough to show β-glucosidase activity on umbelliferone.

The method of Lowry, et al. (1951) was used to determine the amount of protein adsorbed to the calcium phosphate gel. The following results (Table 4) were obtained by taking optical density readings on the spectrophotometer at 650 nm and extrapolating the µg/ml from the standard graph (Figure 2) plotted from known concentrations of human serum albumin:

TABLE 4

PROTEIN DETERMINATION

Treatment	Electrophoretic Pattern	O.D. (650 nm)	g/ml	O.D. (410 nm)
dialyzed conidial wash	"W_1" + "Y" (Class 3)	0.315	350	0.872
supernatant portion of gel-treated conidial wash	"W_1" (Class 4)	0.155	150	0.222

From these figures, I concluded that 57% of the protein in the conidial wash was adsorbed by the calcium phosphate gel. A PNPG assay (at 410 nm) showed that 75% of the β-glucosidase was adsorbed. Although it is not known exactly how much "W_1" was adsorbed with "Y", 25% of the β-glucosidase remained in the supernatant fraction after the calcium phosphate gel adsorption, and it was visibly (by electrophoresis) all "W_1". The

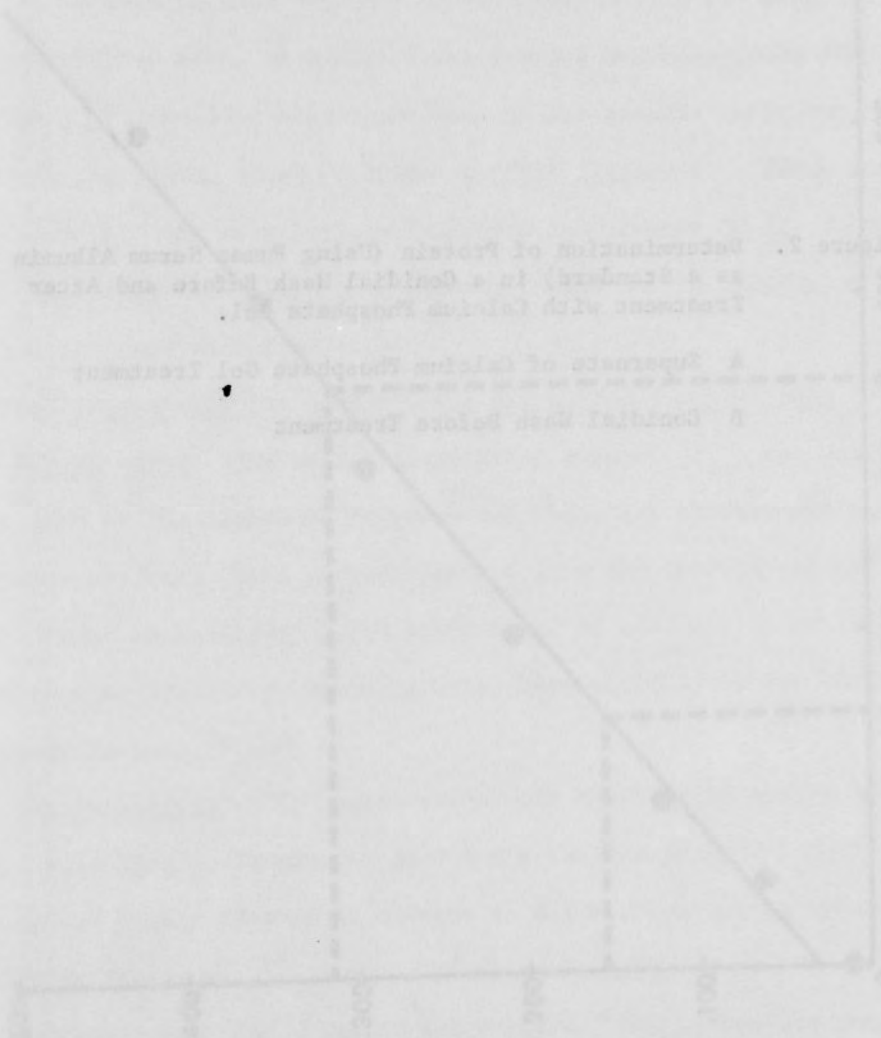
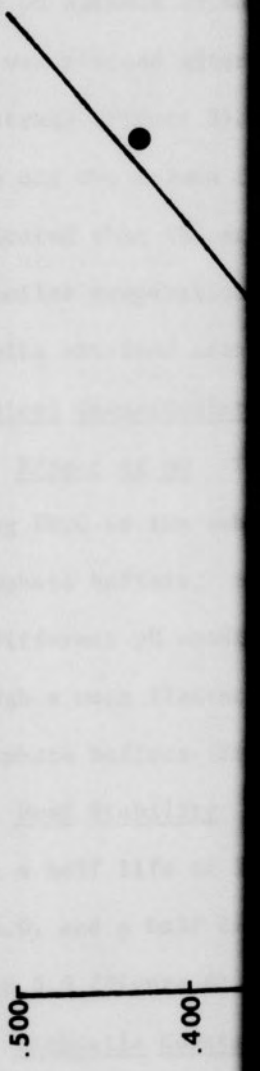
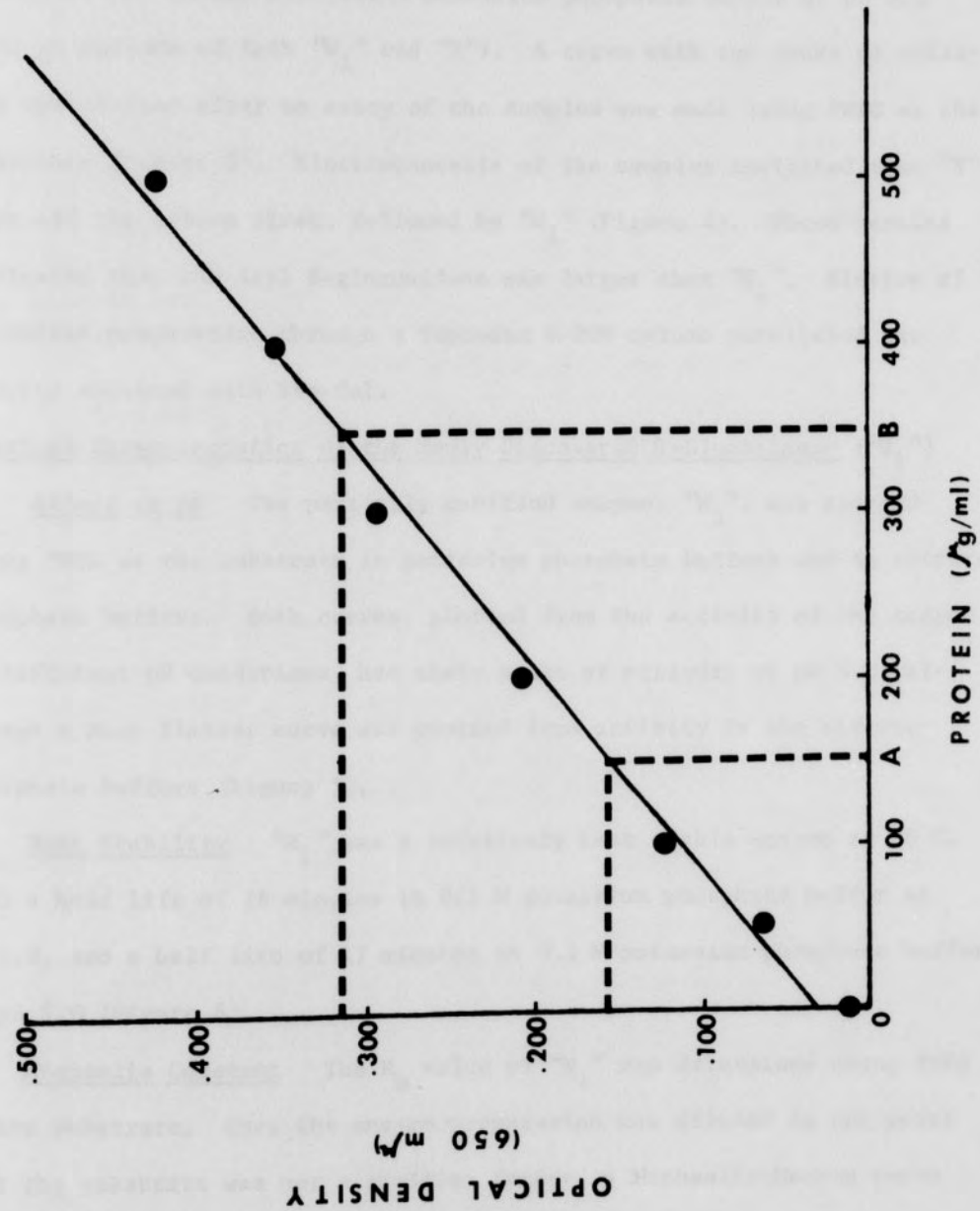


Figure 2. Determination of Protein (Using Human Serum Albumin as a Standard) in a Conidial Wash Before and After Treatment with Calcium Phosphate Gel.

A Supernate of Calcium Phosphate Gel Treatment

B Conidial Wash Before Treatment




OPTICAL DENSITY
(650 mμ)
.500
.400
.300
.200
.100
0
100
A
200
300
B
400
500
PROTEIN (μg/ml)

supernatant fraction, containing partially purified "W_1", was concentrated and used in determining the physical characteristics of "W_1".

A concentrated, conidial wash preparation of P-212 was eluted on a Bio-Gel P-200 column with 0.1 M potassium phosphate buffer at pH 5.0 (the pH optimum of both "W_1" and "Y"). A curve with two peaks of activity was plotted after an assay of the samples was made using PNPG as the substrate (Figure 3). Electrophoresis of the samples indicated that "Y" came off the column first, followed by "W_1" (Figure 4). These results indicated that the aryl β-glucosidase was larger than "W_1". Elution of a similar preparation through a Sephadex G-200 column paralleled the results obtained with Bio-Gel.

Physical Characteristics of the Newly Discovered β-Glucosidase ("W_1")

Effect of pH The partially purified enzyme, "W_1", was assayed using PNPG as the substrate in potassium phosphate buffers and in citrate phosphate buffers. Both curves, plotted from the activity of the enzyme at different pH conditions, had their peaks of activity at pH 5.0, although a much flatter curve was plotted from activity in the citrate phosphate buffers (Figure 5).

Heat Stability "W_1" was a relatively heat stable enzyme at 60 C, with a half life of 16 minutes in 0.1 M potassium phosphate buffer at pH 6.0, and a half life of 17 minutes in 0.1 M potassium phosphate buffer at pH 5.0 (Figure 6).

Michaelis Constant The K_m value of "W_1" was determined using PNPG as the substrate. Once the enzyme preparation was diluted to the point that the substrate was not a limiting factor, a Michaelis-Menton curve was plotted (Figure 7). Later, the data was plotted according to the

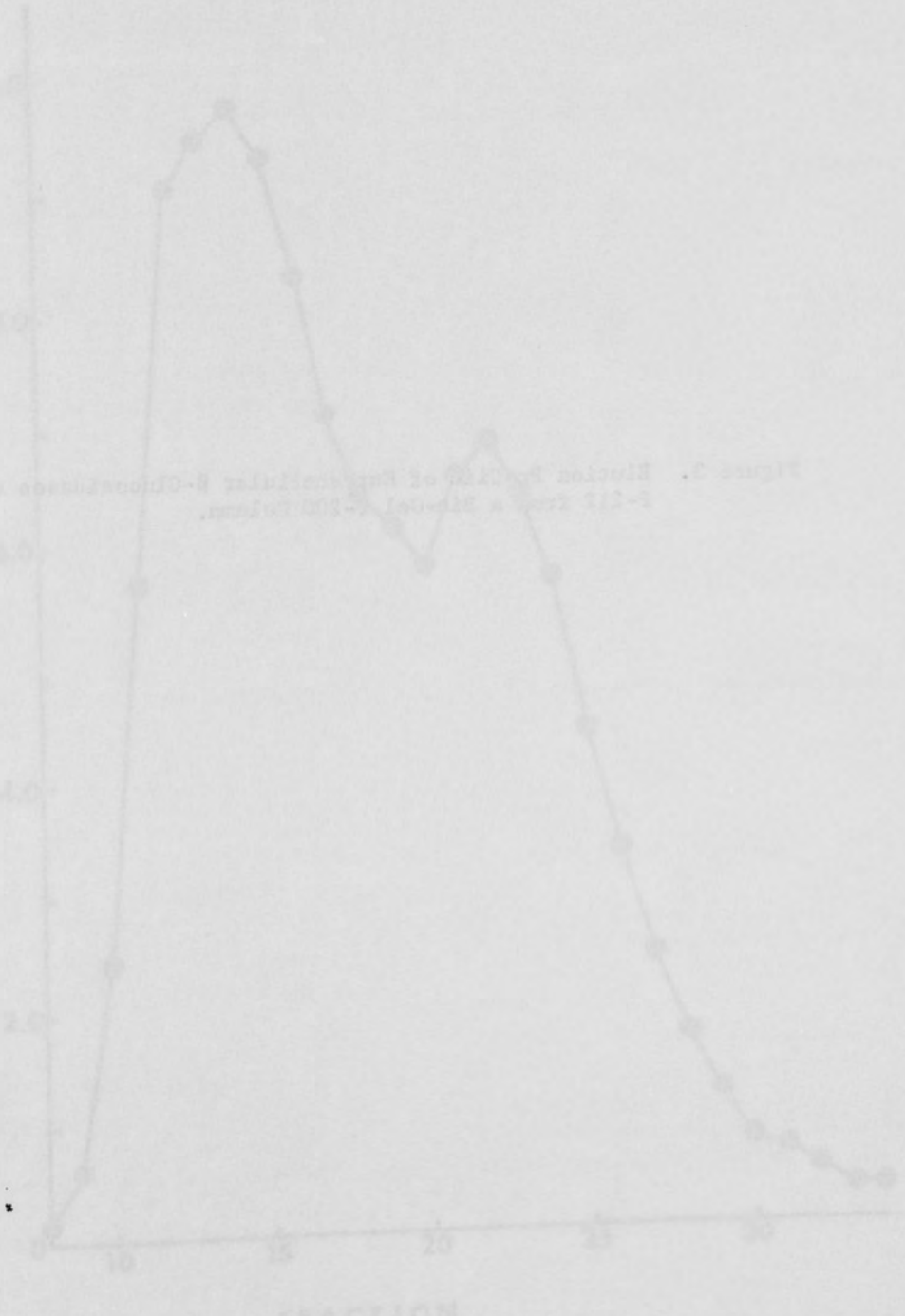
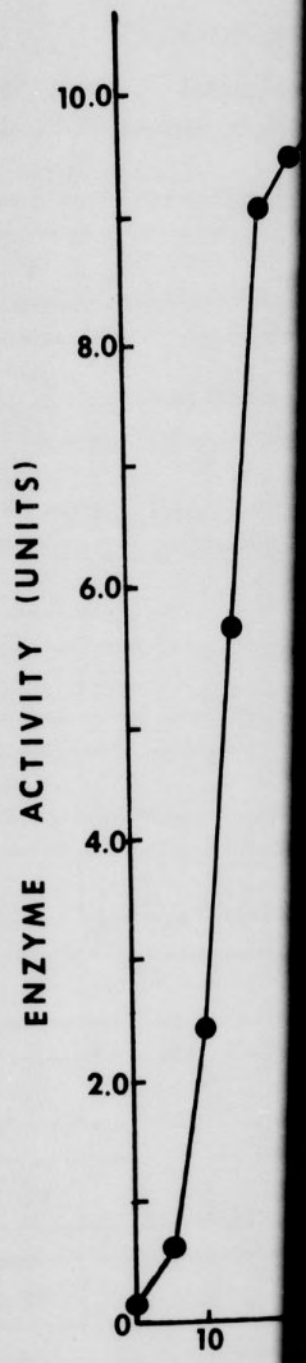
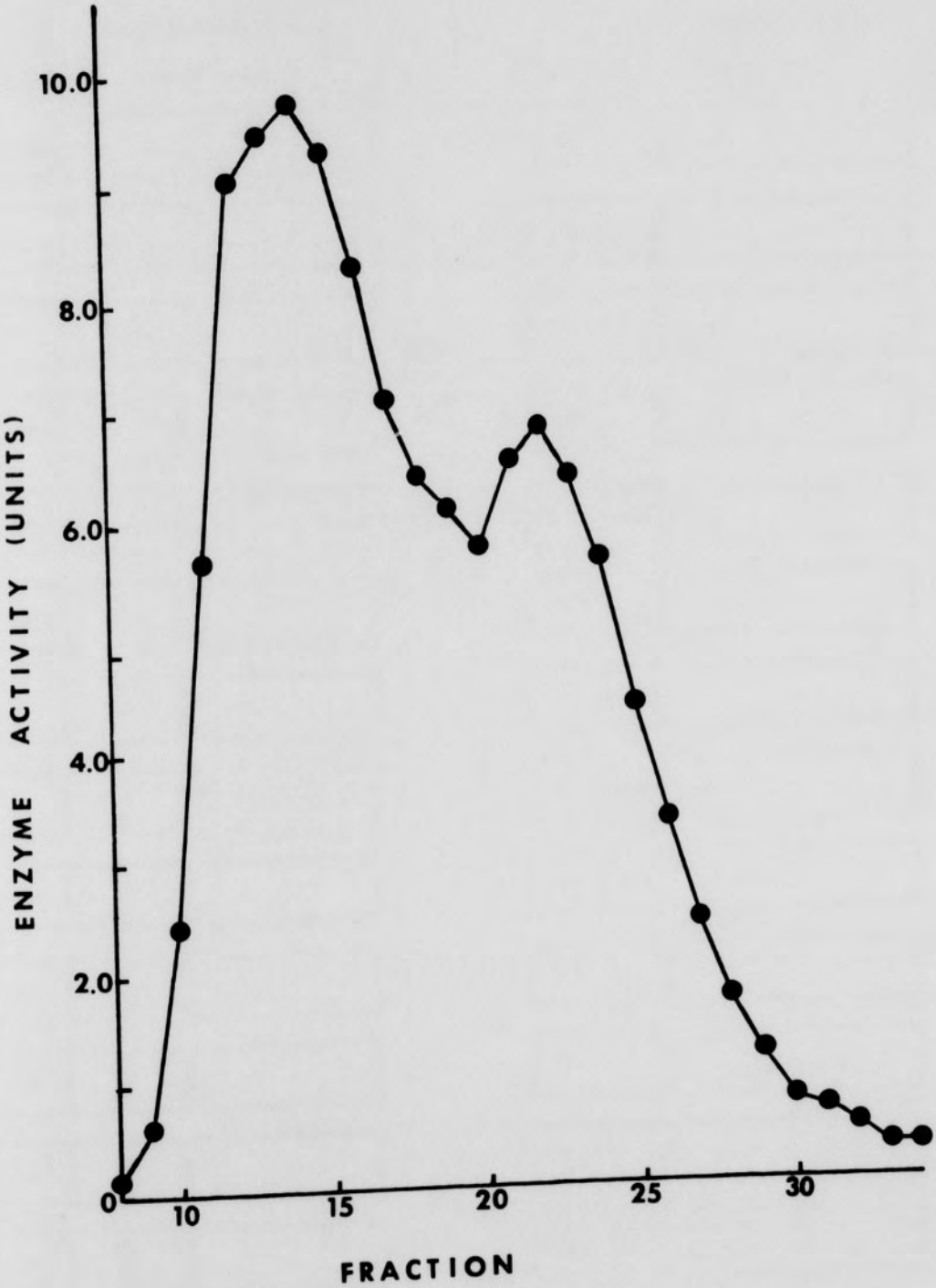


Figure 3. Elution Profile of Extracellular β-Glucosidases of P-212 from a Bio-Gel P-200 Column.




10.0
8.0
6.0
4.0
2.0
0
10
15
20
25
30
ENZYME ACTIVITY (UNITS)
FRACTION

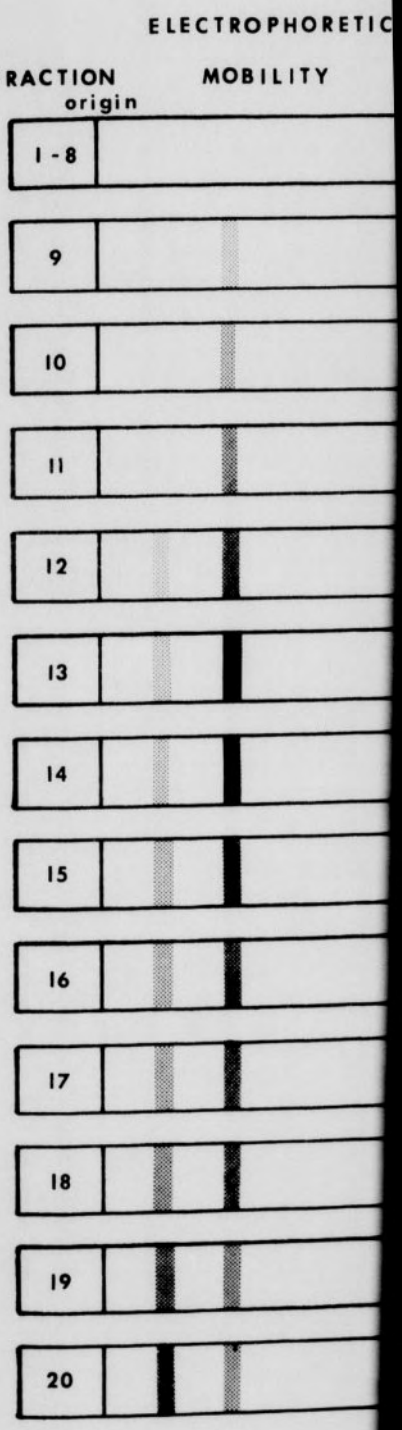
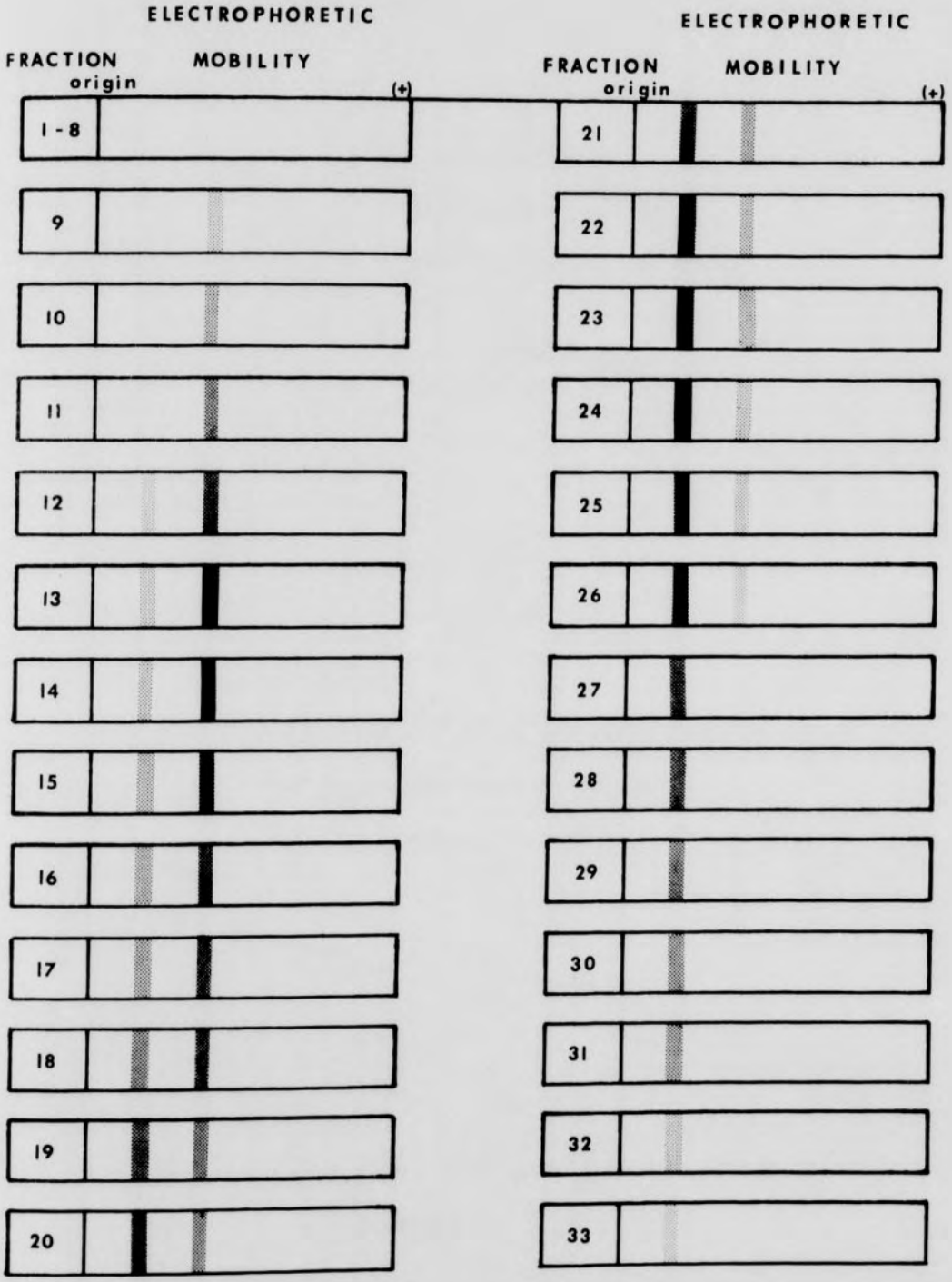


Figure 4. Electrophoretic Patterns of 1 ml Fractions After Elution of P-212 Conidial Wash from Bio-Gel P-200 Column.


ELECTROPHORETIC
MOBILITY
FRACTION
origin
(+)
1 - 8
9
10
11
12
13
14
15
16
17
18
19
20
ELECTROPHORETIC
MOBILITY
FRACTION
origin
(+)
21
22
23
24
25
26
27
28
29
30
31
32
33

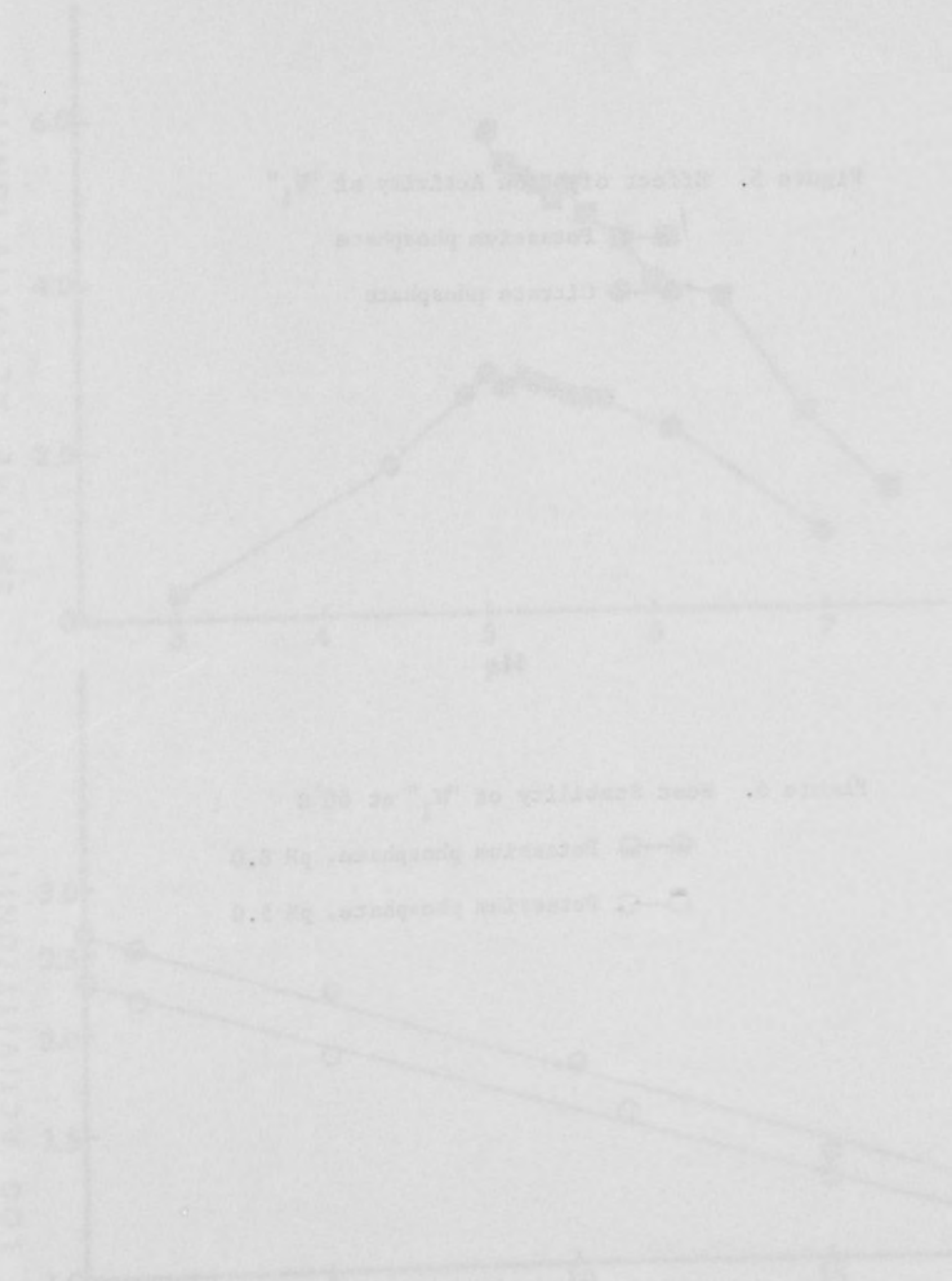


Figure 5. Effect of pH on Activity of "W_1"

■—■ Potassium phosphate

●—● Citrate phosphate

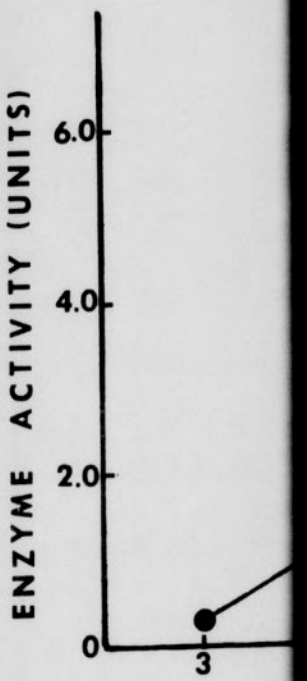
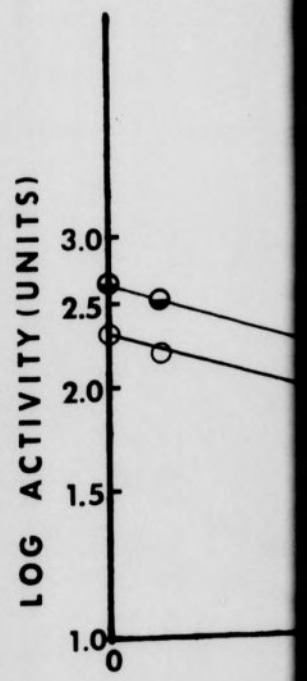
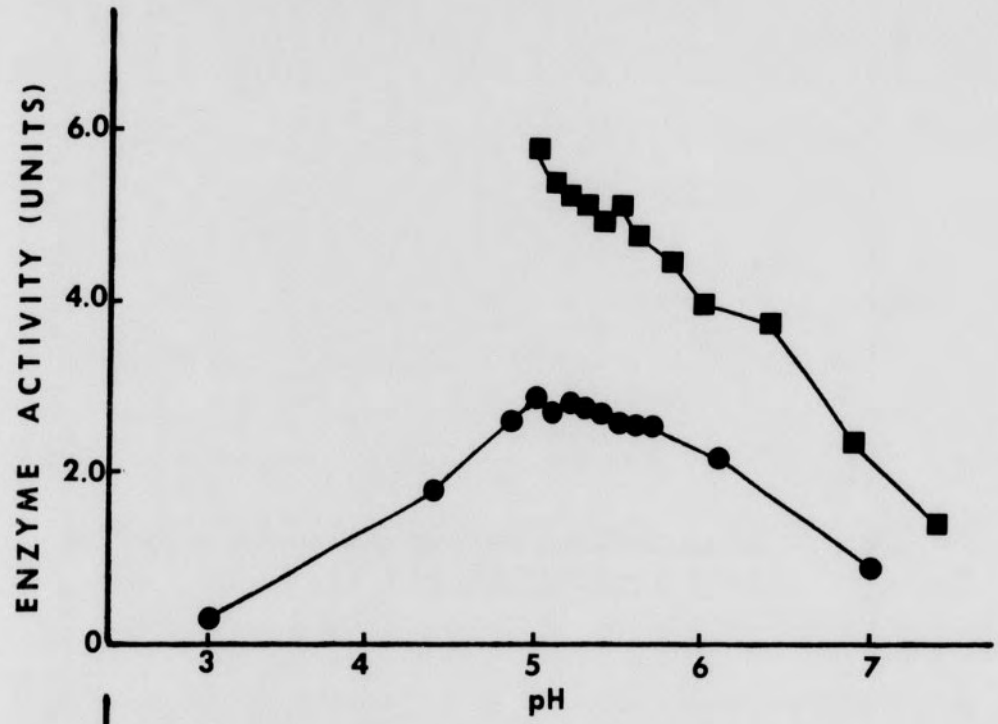


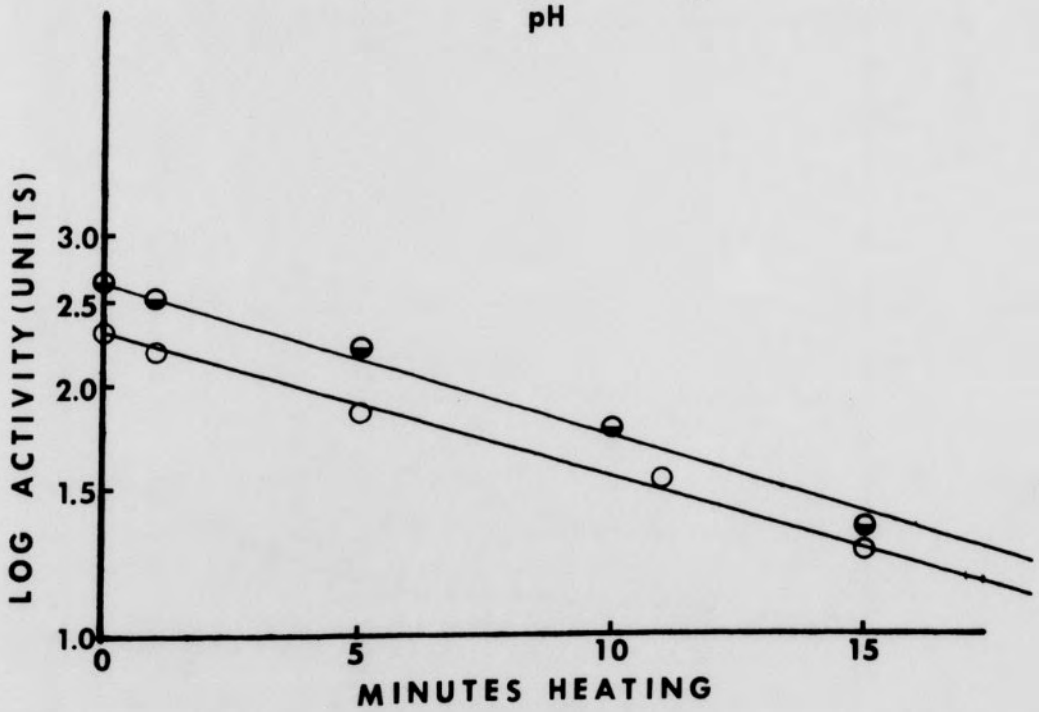
Figure 6. Heat Stability of "W_1" at 60 C

◐—◐ Potassium phosphate, pH 6.0

○—○ Potassium phosphate, pH 5.0




ENZYME ACTIVITY (UNITS)
6.0
4.0
2.0
0
3
4
5
6
7
pH


LOG ACTIVITY (UNITS)
3.0
2.5
2.0
1.5
1.0
0
5
10
15
MINUTES HEATING

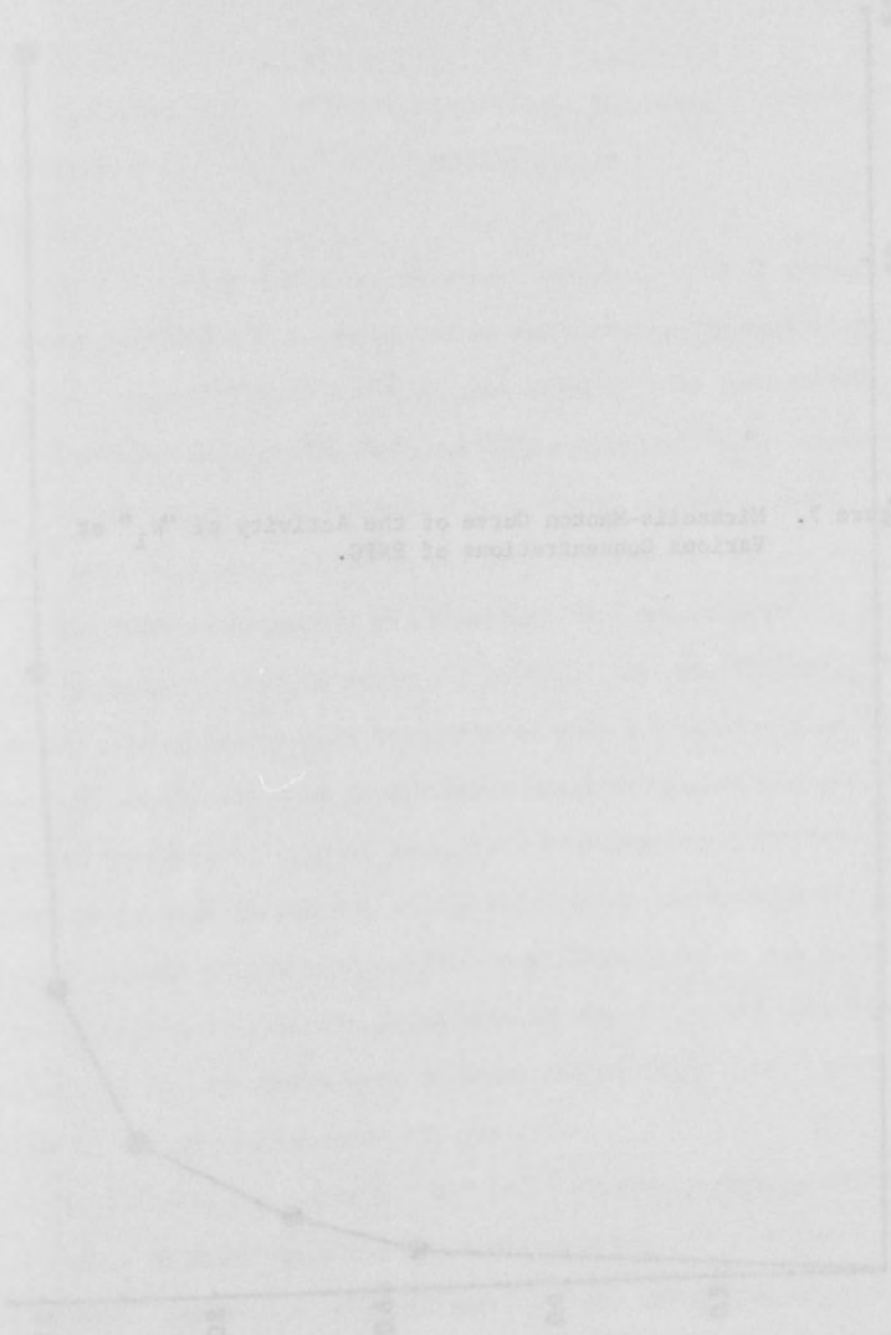
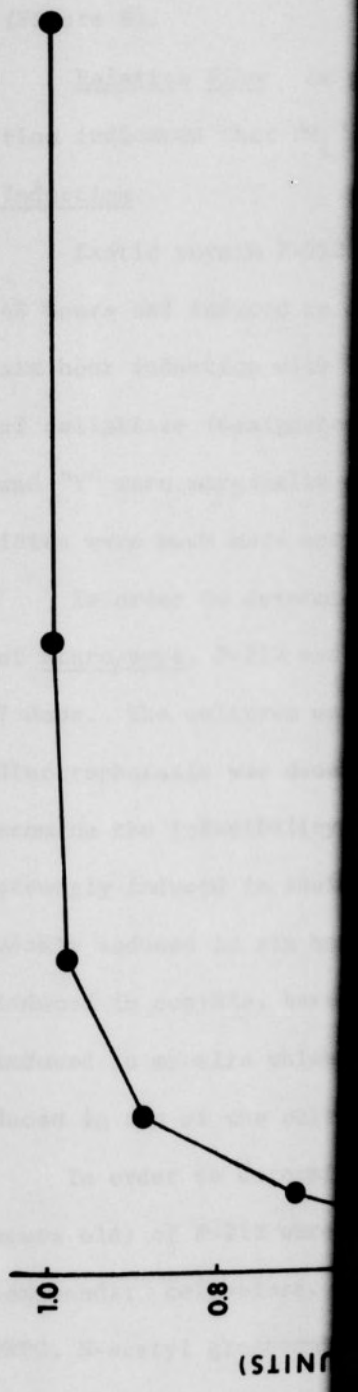
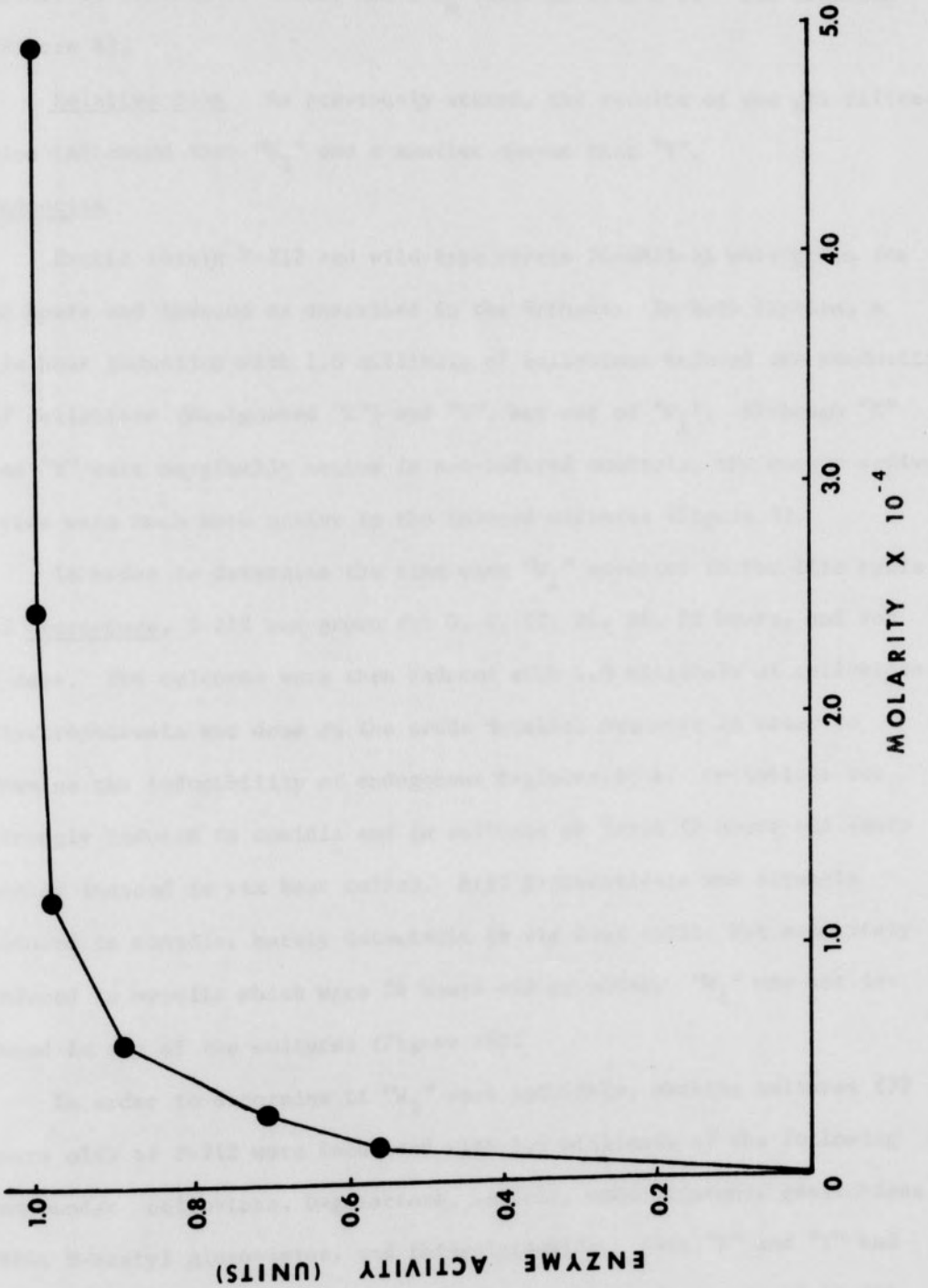


Figure 7. Michaelis-Menton Curve of the Activity of "W_1" at Various Concentrations of PNPG.




ENZYME ACTIVITY (UNITS)
1.0
0.8
0.6
0.4
0.2
0
1.0
2.0
3.0
4.0
5.0
MOLARITY X 10^{-4}

method of Lineweaver-Burke, and a K_m value of 1.98×10^{-5} was obtained (Figure 8).

Relative Size As previously stated, the results of the gel filtration indicated that "W_1" was a smaller enzyme than "Y".

Induction

Exotic strain P-212 and wild-type strain 74-OR23-1A were grown for 48 hours and induced as described in the Methods. In both strains, a six hour induction with 1.0 millimole of cellobiose induced the production of cellobiase (designated "X") and "Y", but not of "W_1". Although "X" and "Y" were marginally active in non-induced controls, the enzyme activities were much more active in the induced cultures (Figure 9).

In order to determine the time when "W_1" occurred in the life cycle of Neurospora, P-212 was grown for 0, 6, 12, 24, 48, 72 hours, and for 7 days. The cultures were then induced with 1.0 millimole of cellobiose. Electrophoresis was done on the crude mycelial extracts in order to examine the inducibility of endogenous β-glucosidase. Cellobiase was strongly induced in conidia and in cultures at least 12 hours old (only weakly induced in six hour cells). Aryl β-glucosidase was strongly induced in conidia, barely detectable in six hour cells, but moderately induced in mycelia which were 24 hours old or older. "W_1" was not induced in any of the cultures (Figure 10).

In order to determine if "W_1" were inducible, shaking cultures (72 hours old) of P-212 were incubated with 1.0 millimole of the following compounds: cellobiose, D-galactose, salicin, umbelliferone, gentiobiose, PNPG, N-acetyl glucosamine, and thiogalactoside. Both "X" and "Y" had weak activity in the non-induced control. Cellobiase was found in all

Figure 8. Lineweaver-Burke Plot of the Activity of "W_1" at Various Substrate Concentrations.

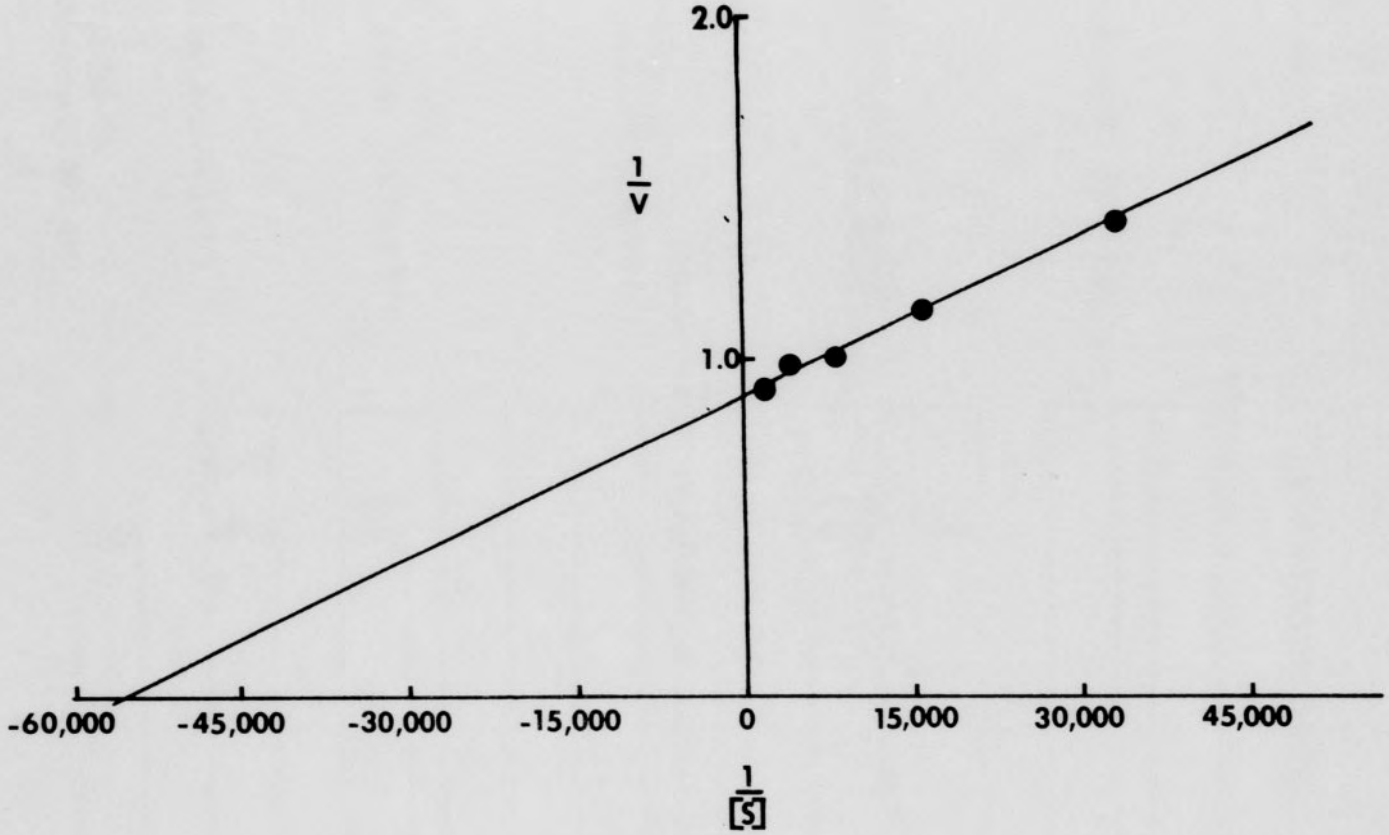
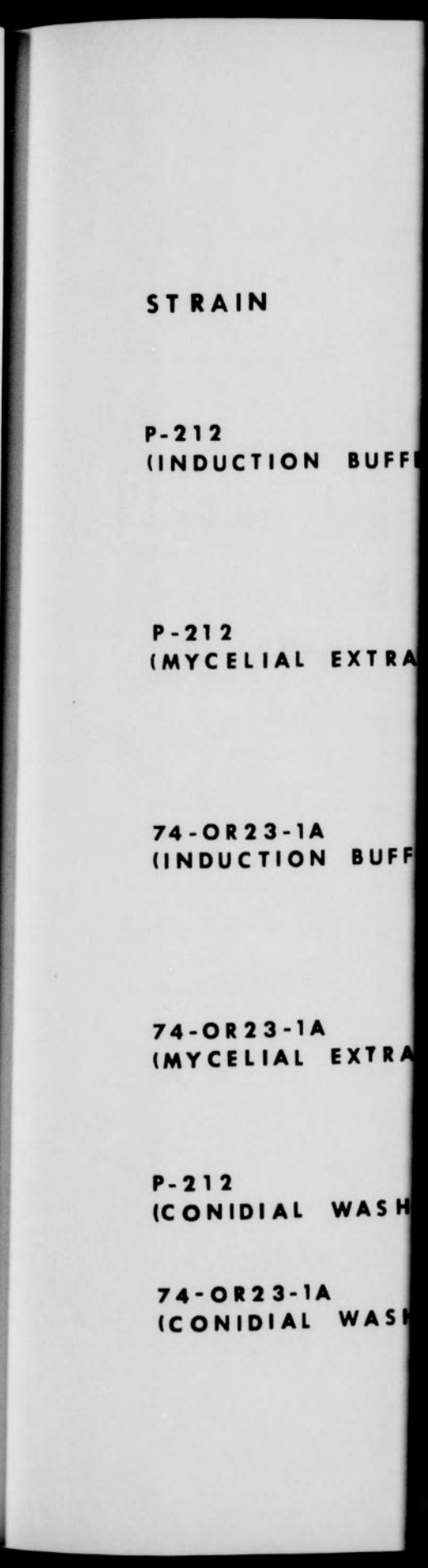

2.0
1.0
1/V
-60,000
-45,000
-30,000
-15,000
0
15,000
30,000
45,000
1/[S]

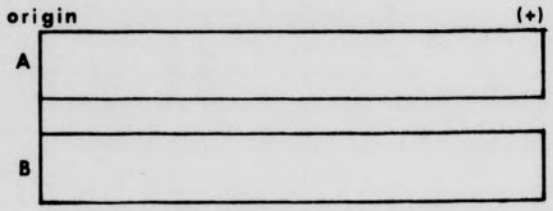
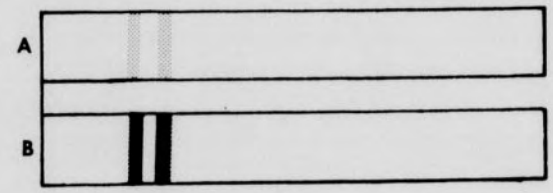
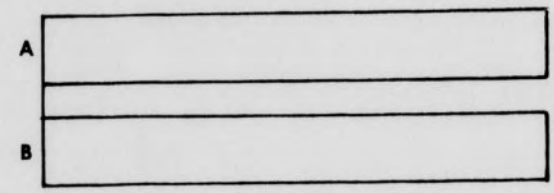
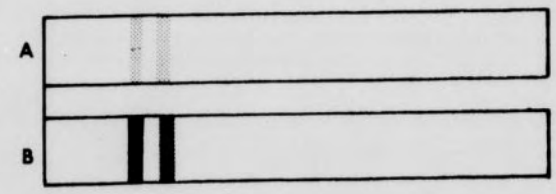
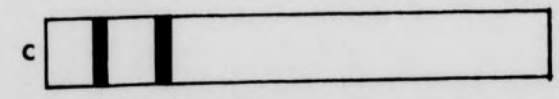
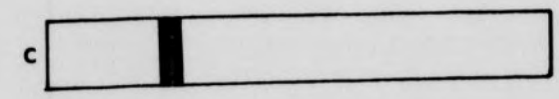
Figure 9. Electrophoretic Patterns Following Induction of P-212 and 74-OR23-1A with Cellobiose.

A Non-induced

B Induced

C Conidial Wash (non-induced)



STRAIN	ELECTROPHORESIS
P-212 (INDUCTION BUFFER)	
P-212 (MYCELIAL EXTRACT)	
74-OR23-1A (INDUCTION BUFFER)	
74-OR23-1A (MYCELIAL EXTRACT)	
P-212 (CONIDIAL WASH)	
74-OR23-1A (CONIDIAL WASH)	

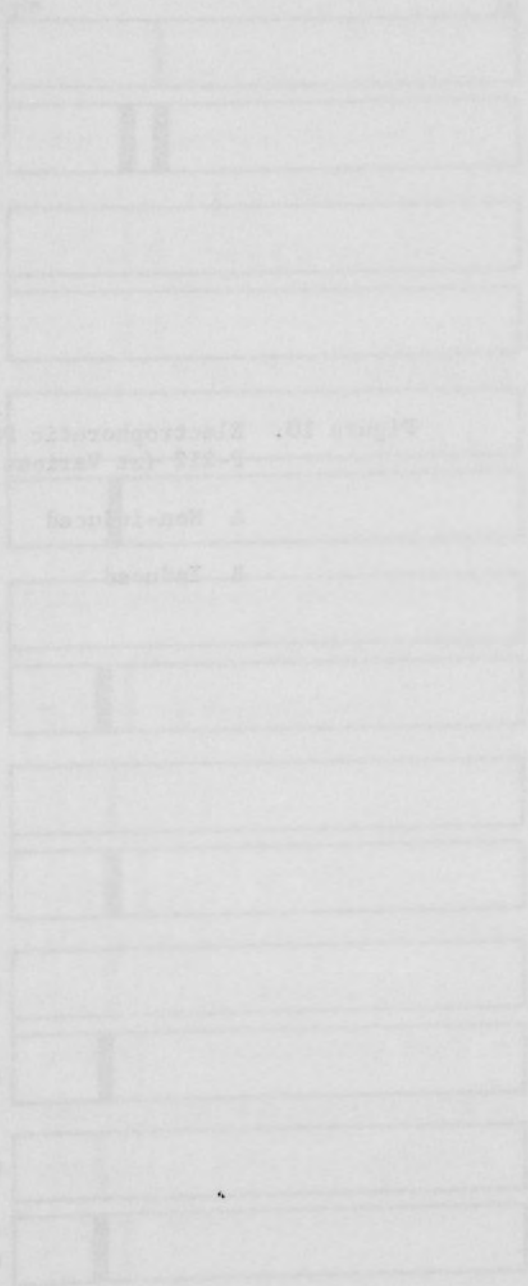


Figure 10. Electrophoretic Patterns Following Induction of P-212 (at Various Stages of Growth) with Cellobiose.

A Non-induced

B Induced

AGE AT INDUCTI

CONIDIA

6 HOUR

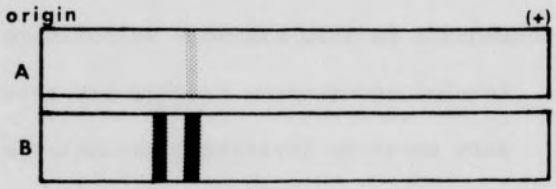
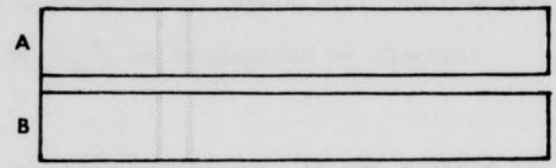
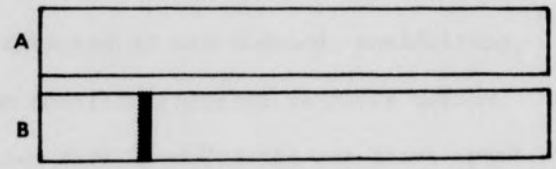
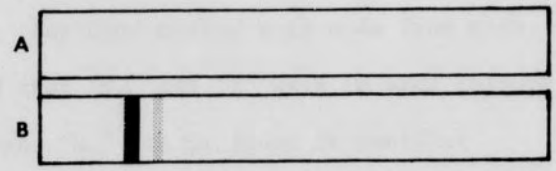
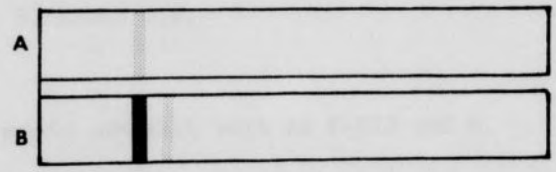
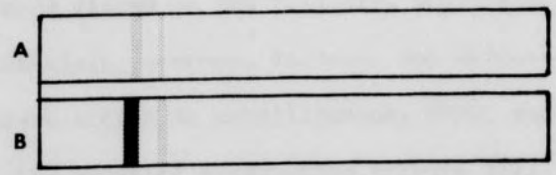
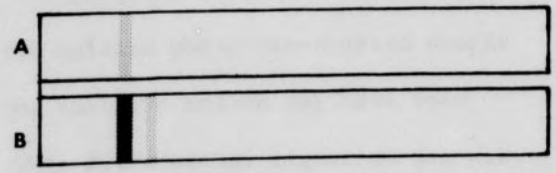
12 HOUR

24 HOUR

48 HOUR

72 HOUR

7 DAY

AGE AT INDUCTION	ELECTROPHORESIS
CONIDIA	
6 HOUR	
12 HOUR	
24 HOUR	
48 HOUR	
72 HOUR	
7 DAY	

of the mycelial extracts, regardless of the inducer. Aryl β-glucosidase was induced only by cellobiose and N-acetyl glucosamine. This atypical reaction of "X" and of "Y" in the presence of inducers will be discussed. The Sepratek method of electrophoresis was used to examine the induced mycelial extracts. The typical electrophoretic patterns observed with this method are shown in Figure 11 (analogous to Figure 1b). No compound was able to induce the production of "W_1", as determined by electrophoresis (Figure 12).

Since "W_1" was not found in any induced cultures, it was decided to determine how early "W_1" could be detected in non-induced, conidiating, still cultures. P-212 was grown on modified glycerol complete medium for three, four, five, six, and seven days (conidiation was first apparent three days after inoculation). Conidial washes were made from each culture. Electrophoresis revealed that "W_1" and "Y" were in each culture. In the presence of conidiation, then, "W_1" can be found in conidial washes from strains which are only 72 hours old.

Substrate Specificity

Electrophoresed samples of a crude conidial wash of P-212 and a partially purified sample of "W_1" were placed on the following substrates: umbelliferone, PNPG, cellobiose, trehalose, sucrose, lactose, and maltose. "W_1" and "Y" in the conidial wash were active on umbelliferone, PNPG, and cellobiose, and "W_1" was active in the purified preparation (Figure 13). "W_1" migrated slightly farther in the calcium phosphate-treated sample than in the conidial wash, indicating that the enzyme may have been slightly altered after treatment. This differential migration was not consistent with earlier electrophoretic runs, in which the electrophoretic

Figure 11. Typical Electrophoretic Patterns of Neurospora β-Glucosidases -- Sepratek System. (analogous to Figure 1b)

Figure 12. Electrophoretic Patterns Following Induction of P-212 with Various Inducers.

CLASS EN

1	Y
2	X
3	W_1
4	W_1
5	Y
6	W_1

INDUCER

NON-INDUCED CO
CELLOBIOSE
PNPG
UMBELLIFERONE
SALICIN
GALACTOSE
N-ACETYL GLUC
THIOGALACTOSI

CLASS	ENZYMES	ELECTROPHORESIS origin (+)
1	Y	I
2	X + Y	II
3	W_1 + Y	I I
4	W_1	I
5	Y + W_2	II
6	W_1 + Y + W_2	I II

INDUCER	ELECTROPHORESIS origin (+)
NON-INDUCED CONTROL	II
CELLOBIOSE	II
PNPG	I
UMBELLIFERONE	I
SALICIN	I
GALACTOSE	I
N-ACETYL GLUCOSAMINE	II
THIOGALACTOSIDE	I

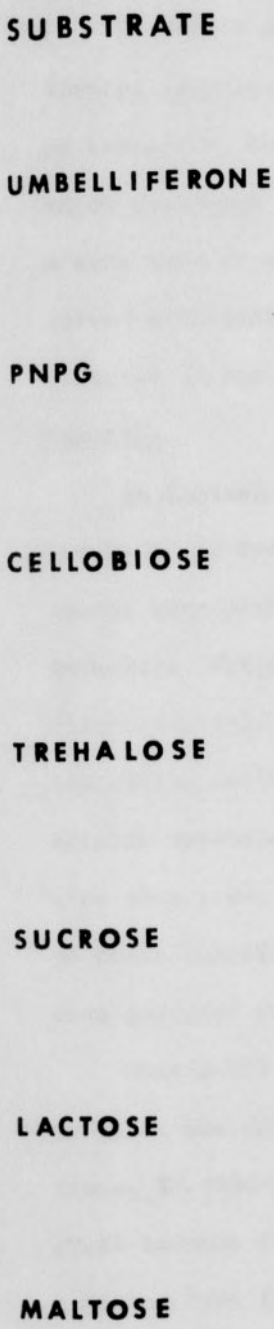


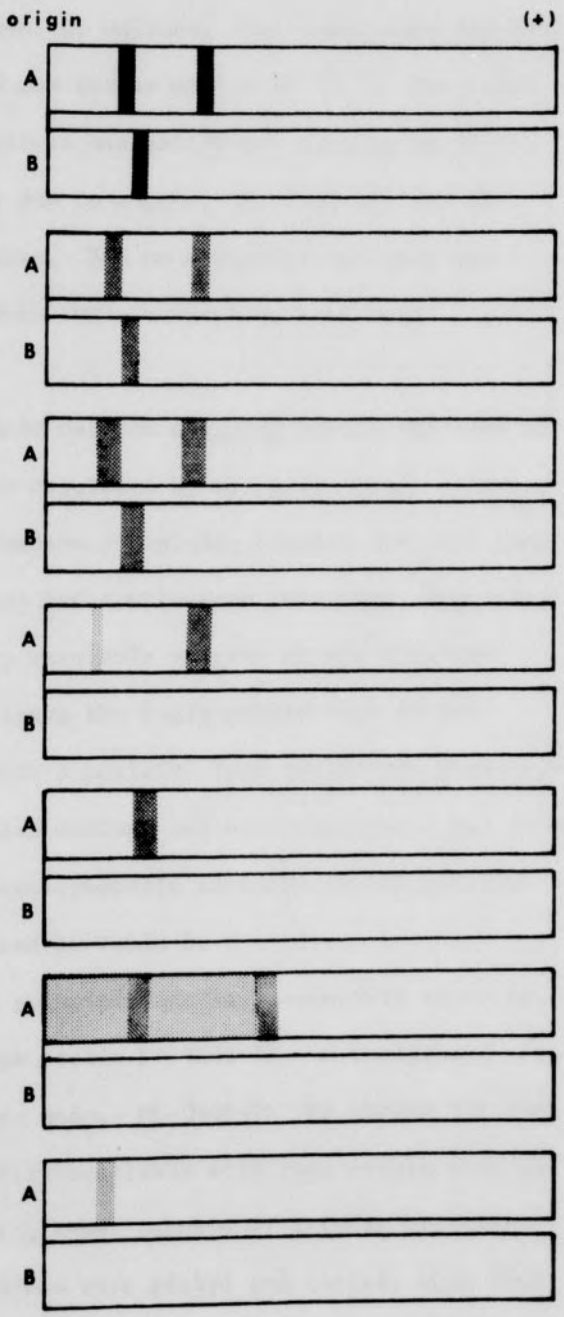
Figure 13. Electrophoretic Patterns from Various Substrates Following Electrophoresis.

A Conidial wash

B Conidial wash after treatment with calcium phosphate gel ("purified W_1")

SUBSTRATE

ELECTROPHORESIS



mobility of "W_1" had been consistant, regardless of the treatment.

Enzymatic activity in the conidial wash was apparent with the substrates trehalose, sucrose, lactose, or maltose. Two bands were observed on trehalose, but the slower band was not as mobile as "W_1". The bands which developed on sucrose and maltose had mobilities similar to "W_1". A wide band of activity, probably due to a group of isozymes, was observed with lactose as the substrate. But no enzymatic activity was detected in the purified sample when these substrates were used.

Genetics

An initial cross (M-1) was made between a gluc-2 strain (RB-1(83)a) and P-212 to determine if "W_1" was regulated by the gluc locus. The spores were picked and tested on medium containing esculin and iron (see Methods). While 50% of the progeny had a wild-type phenotype (β-glucosidase activity), 50% of the progeny were able to grow on the esculin-containing medium but unable to cleave the β-glucosidic bond in the esculin molecules. The strains with a positive test (wild-type phenotype) were then grown on glycerol complete medium, and electrophoresis was done on their conidial washes. The electrophoretic patterns of the esculin-iron positive progeny are summarized in Table 5.

Once P-212 was chosen as the principle exotic strain with which to work, it was crossed with wild-type strain NC-OR66-1a. A reciprocal cross, NC-OR66-1a X P-212, was also made. NC-OR66-1a was chosen for the cross because it was the only readily available wild-type strain with an a mating type (P-212 had an A mating type) capable of forming protoperithecia. The spores from these crosses were picked and tested, with 100% positive results, on esculin-containing medium (Table 5).

TABLE 5

GENETICS OF β-GLUCOSIDASES

CROSS	PARENTS: MATERNAL (electrophoretic pattern)	PARENTS: PATERNAL (electrophoretic pattern)	SPORES PICKED	% GERMINATION	% ES-FE +	ELECTROPHORETIC PATTERNS (Fig. 1)					
						W_1	-	-	+	+	-	-
						X	-	+	-	-	-	-
						Y	+	+	+	-	+	+
						W_2	-	-	-	-	+	+
							1	2	3	4	5	6
M-1	RB-1(83)a (gluc-2)	P-212 (class 3)	50	36	50%		0	0	3	0	2	4
M-2	NC-OR66-1a (class 5)	P-212 (class 3)	100	16	100%		0	0	10	0	2	4
M-3	P-212 (class 3)	NC-OR66-1a (class 5)	100	28	100%		4	0	8	0	6	8
M-5	M-3(17) (class 6)	74-OR8-1a (class 5)	50	46	-		0	0	0	0	10	13
M-6	M-2(12) (class 3)	74-OR8-1a (class 5)	50	44	-		0	0	6	0	5	11
M-7	M-3(9) (class 3)	74-OR8-1a (class 5)	50	58	-		0	0	7	0	8	14

Since the germination ratio and the number of strains analyzed were very low in the first generation crosses, second generation crosses were made back with wild-type strains, in an attempt to integrate the "W_1" phenomenon into wild-type stock. But the activity of "W_1" seemed to diminish in the later generations. In P-212, the activity of "W_1" was generally greater on umbelliferone (as observed by greater fluorescence) than that of "Y". In first generation progeny, "W_1" and "Y" seemed to have similar levels of activity. However, in the second generation, "W_1" was often very much less active than "Y", often requiring several minutes to half an hour for fluorescence to be observed. This effect could possibly be due to the action of modifiers.

Occurrence of the New β-Glucosidase ("W_1")

After "W_1" was found in the exotic strains, it was found in three other, unrelated, strains. It was observed in the conidial washes of two gluc-1 strains, 33(3-7) and 22(2-5), and in a mycelial extract of gluc-2 strain, CM-62(2-2)a. The mycelial extract had been treated with ammonium sulfate as described in the Methods, and it was the source for the known "X" control. Electrophoresis revealed that "X" was the only β-glucosidase (or at least the only one concentrated enough to yield visible activity) in the 0 - 40%, 40 - 50%, and 60 - 65% fractions, and in the initial unsalted extract. Both "W_1" and "Y" were found in the 50 - 60% fraction. When the 50 - 60% fraction was heated at 60 C for one minute, "X" was inactivated (thermolabile enzyme), but the band at the "W_1" position retained enough activity to be assayed by electrophoresis. When the 50 - 60% fraction was heated at 100 C for one minute, the "W_1" band, as well as "X", was inactivated; indicating that the

activity found at the "W_1" position must also be enzymatic. A possible explanation of these findings will be discussed.

Discovery of a Second New β-Glucosidase ("W_2")

After finding that a conidial preparation could be electrophoresed without being concentrated first, I harvested the conidia directly out of test tubes. Only 2 ml of glass distilled water was added to each tube (Table 2), followed by centrifugation at 17,300 X g for 15 minutes. When the samples were placed in an electrical field, it was found that enzymatic activity was strong enough to adequately hydrolyze the umbelliferone substrate. Two bands were apparent after electrophoresis. One band had the electrophoretic mobility of "Y", but the second band migrated slightly farther than "Y". The faster band was designated "W_2", and the electrophoretic pattern of "Y" and "W_2" is denoted Class 5 in Figure 1. Aryl β-glucosidase and "W_2" will be referred to as "the doublet" because of their close proximity following electrophoresis.

Preliminary data gathered on "W_2" indicated that it was inactivated when heated at 100 C for one minute, and that its hydrolytic activity increased over time. This enzyme was never reported before because early electrophoresis experiments employed esculin or PNPG as the substrate. Strains in which the "doublet" had appeared were re-examined with esculin and PNPG as substrates. Instead of two bands, one faint, fuzzy band was observed at the location of "the doublet". It appeared that the efficiency of "W_2" on umbelliferone was high, permitting an immediate reaction, which could be scored before diffusion obscured the slight separation. Quenching of fluorescence in esculin was less sensitive, and the separation was not visible. The chromogenic substrate,

PNPG, involved a ten minute reaction before the addition of Tris, and by this time, no separation could be detected.

Further concentration of the crude preparation, of ammonium sulfate to the sample, or decreasing or increasing the age of the culture before harvest had no effect on the presence of "the doublet". In fact, repeated electrophoresis of a variety of wild-type strains and exotics revealed that the "doublet" was a common phenomenon. It was found in the conidial washes of all of the wild-type strains tested, and in about half of the exotic strains.

Strains which had previously demonstrated "W_1" and "Y" (Class 3 electrophoretic pattern) were grown in Erlenmeyer flasks (varying in size from 125 ml to 2000 ml) and in test tubes. With only one or two exceptions, "W_1" was enzymatically active only when the strain was grown in a flask. In the instance(s) where "W_1" was found from a strain grown in a tube, its activity was very weak relative to "Y".

Since P-212 inoculated, from a tube which lacked "W_1" in the conidial wash, into a flask, possessed "W_1" in its conidial wash; it seemed advisable to determine if the presence or absence of "W_1" and/or "W_2" was due to some variable growth condition such as aeration, humidity, surface area, or concentration of nutrients. P-212 was inoculated into 500 ml Erlenmeyer flasks containing 25, 50, and 100 ml of modified glycerol complete medium. Conidia were then inoculated into three 500 ml flasks containing 50 ml of medium. One of the flasks was incubated in front of a fan; one of the flasks had aluminum foil taped over the cotton plug; and one was incubated as a control. A conidial wash was made from each flask with the result that "W_1" and "Y" were active

under each condition. It was noted, however, that the amount of growth, conidiation, and production of exogenous β-glucosidases was proportional to the amount of medium present and to the amount of aeration.

DISCUSSION

The research in this thesis, involving the biochemical and genetic characterization of an isozyme ("W_1" in the β-glucosidase system of Neurospora crassa, will be discussed under several headings.

Historical Parallels

The β-glucosidase isozyme system has several parallels in Neurospora and in other fungi. Bates, Hedman, and Woodward (1967), Johnson (1967), and Johnson and DeBusk (1970a,b) have described the β-galactosidase system, which includes a large enzyme with a pH optimum of 7.5 and two smaller enzymes with pH optima at 4.2 (Landman and Bonner, 1952; Landman, 1954) and at 4.5 (Johnson, 1969). Multiple forms of invertase have been studied by Metzenberg (1962, 1963, 1964) and by Trevithick and Metzenberg (1964, 1966) and are thought to be due to active subunits (which are thermostable) and to the predominate aggregate form. Several investigators (Horowitz and Fling, 1953; Horowitz, 1956; Horowitz, et al., 1960, 1961) have described tyrosinase isozymes with varying thermostabilities, and they have elucidated the induction and genetic control of this system. The multiple forms of trehalase have been described by Hill and Sussman (1963, 1964), and later the enzymes were found to be associated with, but not necessarily the cause of, conidiation (Hanks and Sussman, 1969a,b).

The multiple forms of β-glucosidase have been separated and characterized in other organisms: in Saccharomyces (Fleming and Duerksen, 1967a,b), in Dictyostelium (Coston and Loomis, 1969), in Alcaligenes

(Han and Srinivasan, 1969), and in Myrothecium (Hash and King, 1958a,b).

Until we are more certain of the role of "W_1" in the β-glucosidase system of Neurospora, it will be difficult to interpret results in terms of other systems. Except where definite parallels exist to other systems and other organisms, the discussion of "W_1" will necessarily be limited to its relation to aryl β-glucosidase and cellobiase and to its regulation and role in a specific isozyme system.

Eberhart and Mahadevan have studied the β-glucosidase system of Neurospora. An exogenous cellulase, an endogenous cellobiase ("X"), and a mural aryl β-glucosidase ("Y") have been separated and characterized. The physical characteristics of "W_1" have been compared (Table 6) with previously determined (Eberhart and Beck, 1970) characteristics of "X" and "Y". But the enzymatic activity of "W_1" could be due to one of many possibilities:

1. It could be a structurally distinct protein with substrate specificity overlapping that of "X" and "Y" (such as acetolacetate synthetase, described by Halpern and Umbarger, 1959).

2. It could differ from "X" and/or "Y" in the proportions of invariant kinds of subunits (such as the lactate dehydrogenase, described by Cahn, et al., 1962).

3. It could be a subunit(s) or a polymer of "X" or "Y" (such as exists with invertase, described by Metzenberg, 1964).

4. It could be due to the association of a carbohydrate or other moiety with the basic enzymatic unit (Jermyn, 1962, described the effect of polysaccharide association with β-glucosidases in Stachybotrys).

5. It could be the degradation product of any of the above, as a result of protease activity.

TABLE 6

PHYSICAL PROPERTIES OF β-GLUCOSIDASES

Property	Cellobiase	Aryl β-glucosidase	W_1
pH optimum	6.1	5.0	5.0
K_m (PNPG)	0.55 mM	1.5 mM	0.0198 mM
K_m (cellobiose)	0.25 mM	6.1 mM	-
thermal half life, 60 C	0.5 min	14-50 min	14-21 min
thermal half life, 46 C	17-50 min	10 hr	-
ammonium sulfate ppt.	55-60%	65-70%	not separated
calcium phosphate gel	-	adsorbed	supernatant fraction
main transglucosylate product from cellobiose	laminaribiose	gentiobiose	-
elution from DEAE, cellulose	0.01 M KCl	0.12 M KCl	-
inducer	cellobiose	cellobiose	none
electrophoretic mobility (mm/30 min, 250 V)	15	19-20	W_1: 9-10 W_2: 20-21
primary location	internal	mural	-
Molecular weight	80,000	168,000	< 168,000
Sephadex G-200	-	early elution	late elution
Bio-Gel P-200	-	early elution	late elution

Relationship of the New Enzyme to Aryl β-Glucosidase and to Cellobiase

From its pH optimum, its extremely high affinity towards PNPG, and its thermal half-life at 60 C, "W_1" could be related to "Y". In fact, "W_1" could be termed a "super" aryl β-glucosidase since it has a much greater affinity (100-fold) for aryl compounds than "Y". The inability to assay "W_1" for its activity on cellobiose, and the low activity of "W_1" on cellobiose following electrophoresis, may indicate that "W_1" has a low affinity (as compared to "X" or "Y") for cellobiose.

Another argument for some sort of relationship to "Y" is the fact that the gluc-2 mutation, which phenotypically results in less than 1% of normal β-glucosidase activity, appears to regulate "W_1" and "Y" simultaneously, without affecting cellobiase. If "W_1" were related to "Y" it could be an active subunit of "Y", since data from the gel filtration indicated that "W_1" was a smaller enzyme than "Y".

The failure to induce the production of "W_1" with any of a variety of substrates, while both "X" and "Y" are easily induced with cellobiose, is a distinct feature of "W_1".

The only evidence for a relatedness between "X" and "W_1" was the discovery of "W_1" in the 50 - 60% fraction of a mycelial extract treated with ammonium sulfate. The strain, CM-62(2-2)a, was a gluc-2 strain which theoretically should have had little detectable "Y" or "W_1" (if, indeed, "W_1" is coordinately regulated by gluc-2) activity. The fact that "W_1" was not found in the original unsalted extract may indicate that "W_1" in this preparation was due to a conversion product. It is also possible, but not yet proven, that "W_1" was present, but not detectable, in the original extract, and that the ammonium sulfate concentrated

the enzyme in the 50 - 60% fraction. This explanation is not very likely since treatment of the conidial wash of P-212 with ammonium sulfate failed to accomplish any significant separation or concentration of "W_1". At this point, it is not known if the "W_1" found in the mycelial extract was identical to, or had the same genetic basis as, the "W_1" found in the conidial wash of exotic strains since "W_1" has not been observed in mycelial extracts of P-212.

It should be apparent that more work needs to be done to determine the origin and regulation of "W_1". Some suggestions as to what could be done are as follows:

1. Determine the size of "W_1", by ultracentrifugation in a sucrose density gradient or by a comparative gel filtration, with compounds of known molecular weight. This should yield more information on whether "W_1" is an active subunit of "Y".

2. Treat "Y" and "X" with various pH conditions, urea, or guanidine HCl in an attempt to cause the conversion of "Y" or "X" to "W_1". Since "W_1" has only been found in conidial washes of exotic strains, one, such as P-212, could be grown in shaking cultures and induced with cellobiose, which would induce the early production of high levels of cellobiase and aryl β-glucosidase activity. After ammonium sulfate precipitation or heat inactivation of "X", the remaining "Y" could then be treated with one of the methods suggested.

3. An immunological study could be done with rabbit antibodies to "Y" ("anti-Y") to determine if "W_1" is CRM+ (positive cross reacting material) with respect to "Y".

Induction

Many questions still remain to be answered concerning the induction studies. Eberhart and Beck (1969) have studied the effect of various inducers on the β-glucosidases. While cellobiose was the most effective inducer for both cellobiase and aryl β-glucosidase, the inducers rarely induced "X" to a greater degree than "Y" using common wild-type stock. When P-212 was treated with a variety of inducers "W_1" was never found to be induced; "X" was induced by every substrate; and "Y" was induced by only two substrates (Figure 10). Being an exotic strain with an extra exogenous β-glucosidase, P-212 has possibly adapted to its present state; and since the enzymes being modified were not necessary for existence, they were relatively unaffected by natural selection. It is apparent that the β-glucosidase system of P-212 will have to be examined further to clarify its unique characteristics and diversities. Two ways in which this could be approached are the following:

1. A conidial wash of P-212 could be treated with calcium phosphate gel. After removal of the supernatant fraction containing "W_1", "Y" could be removed from the gel, using different pH conditions, and characterized to see if it is phenotypically like the "Y" in common wild-type strains.

2. In addition to examining the mycelial extract and the induction buffer for the production of "W_1", perhaps the growth medium should also be examined for early non-induced production of the enzyme.

Although Eberhart and Beck (1969) have shown optimum β-glucosidase induction with cellobiose on 48 - 72 hour cultures for 6 - 8 hours, it is possible (although not very probable) that "W_1" could prove to be

inducible if left in the presence of inducer for a longer period of time.

"W_1" was not found in induced shaking cultures that had few, if any, conidia, varying in age from conidia to seven days old, but it has been found in conidial washes from strains which were only three days old (the third day after inoculation was the first day of conidiation). This may indicate that "W_1" is associated with conidiation, and it may parallel the report by Hill and Sussman (1964) and Hanks and Sussman (1969a,b) that trehalase activity is associated with, but not necessarily the cause of, conidiation. If "W_1" were associated with conidiation, it could be advantageous to a strain where a certain substrate was limited. A highly specific enzyme in such a situation would be an advantageous adaptive mechanism.

Substrate Specificity

From the data in Figure 11, it can be seen that the conidial wash of P-212 contains β-glucosidase activity on umbelliferone, PNPG, and cellobiose and α-glucosidase activity on trehalose and maltose, as well as sucrase and lactase activity. Eberhart (1961) reported finding aryl β-glucosidase, cellobiase, maltase, and invertase, but not lactase, in the conidial extracts of Neurospora. Enzymatic activity at the location of normal "W_1" migration was found in the calcium phosphate gel treated preparation, as well as in the crude conidial wash, when β-glucosides, umbelliferone, PNPG, and cellobiose were the substrates. While affinity of "W_1" for cellobiose was apparently low, it would seem that cellobiose should be able to induce the early production of "W_1". Although no compound has been found to induce the production of "W_1", it would be premature to conclude that "W_1" was definitely not inducible, as it is

possible that induction could occur under different conditions.

Maltase, trehalase, invertase, and lactase activity, although present in the conidial wash, were absent from the purified preparation. It seems reasonable to conclude that these enzymes, along with aryl β-glucosidase, were absorbed to the calcium phosphate gel, and that the specificity of "W_1" is limited to β-glucosides. However, it is possible that "W_1" is active on disaccharides with an α-linkage, but that its activity is not apparent by this method. Use of chromatography (Berger and Eberhart, 1961; Mahadevan, 1963; Jermyn, 1952; Hash and King, 1958a; and Murti and Stone, 1960) with a concentrated, purified "W_1" preparation might produce more conclusive results on the products resulting from the activity of "W_1".

Detection of the New β-Glucosidase

From conditions tested so far, it appears that "W_1" is not extremely sensitive to aeration, humidity, or light. Why then is it rarely observed in culture tubes? Since detection of "W_1" is dependent on electrophoresis, it is conceivable that "W_1" has been produced in culture tubes, but that the enzyme was too dilute to be visibly active on a substrate in the time span in which they were analyzed. Ideally, a quantitative, specific, repeatable assay for "W_1" would be desired. While PNPG provides such an assay, it cannot be used directly since aryl β-glucosidase is assayed at the same time, and the effect is probably additive. The only method of separation is with calcium phosphate, but since it is not known what proportion of "W_1" is adsorbed to the gel or how much dilution of the enzyme occurs, an assay of the supernatant fraction is not very quantitative.

Although "W_1" has never been observed in the conidial wash of a standard wild-type strain, the fact that it has been found in two gluc-1 strains (75-fold concentration) suggests that "W_1" may be found in other strains if the conidial washes were highly concentrated. Concentration of calcium phosphate gel-treated conidial washes of standard wild-type strains might reveal the presence of "W_1" in an even greater variety of strains.

Discussion of a Second New β-Glucosidase ("W_2")

Although a survey of many unrelated strains has revealed the presence of "W_2", attempts to separate "W_2" from "Y" have been unsuccessful. Electrophoretically, "Y" is closely associated with "W_2", which migrated slightly farther. As stated previously, the detection of "W_2" is dependent on electrophoresis at pH 6.0, followed by immediate observation of activity on a highly sensitive substrate, such as umbelliferone.

From indirect evidence, it appears that "W_2" may be related to "Y". "W_2" has never been found in the absence of "Y", and any treatment (such as heat, ammonium sulfate, or calcium phosphate gel) affecting "Y" also affects "W_2". "W_2" seems to be regulated by gluc-2, along with "W_1" and "Y".

"W_2", like "W_1", has never been found in a mycelial extract before or after induction with cellobiose. Its common presence in conidial washes may be evidence that "W_2" activity differs only slightly from "Y". For instance, Jermyn (1962) has shown that many isozymes are due to the association of a carbohydrate or other moiety with the fundamental "Y" protein. The detection of "W_2" in the exogenous wash, and not in mycelial extracts, may indicate that a carbohydrate from the cell wall has

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Discussion of a Second New β-Glucosidase ("W_2")

Although a survey of many unrelated strains has revealed the presence of "W_2", attempts to separate "W_2" from "Y" have been unsuccessful. Electrophoretically, "Y" is closely associated with "W_2", which migrated slightly farther. As stated previously, the detection of "W_2" is dependent on electrophoresis at pH 6.0, followed by immediate observation of activity on a highly sensitive substrate, such as umbelliferone.

From indirect evidence, it appears that "W_2" may be related to "Y". "W_2" has never been found in the absence of "Y", and any treatment (such as heat, ammonium sulfate, or calcium phosphate gel) affecting "Y" also affects "W_2". "W_2" seems to be regulated by gluc-2, along with "W_1" and "Y".

"W_2", like "W_1", has never been found in a mycelial extract before or after induction with cellobiose. Its common presence in conidial washes may be evidence that "W_2" activity differs only slightly from "Y". For instance, Jermyn (1962) has shown that many isozymes are due to the association of a carbohydrate or other moiety with the fundamental "Y" protein. The detection of "W_2" in the exogenous wash, and not in mycelial extracts, may indicate that a carbohydrate from the cell wall has

associated with "Y" to change the structure slightly, which is apparent in electrophoresis.

"W_2", like "W_1", must be isolated to determine its size, physical characteristics, carbohydrate and amino acid content, and substrate affinity.

Genetics of the New β-Glucosidases

It is obvious from the data in Table 5 that any conclusions drawn from such small samples would be questionable and dependent on repeatability in future samples.

In the M-1 cross (a gluc-2 X wild-type strain containing "Y" and "W_2"), 50% of the progeny had a positive test on esculin-iron and 50% had a negative result. This would indicate that the gluc-2 phenotype is due to one gene which segregates independently with its wild-type allele, and that "W_1" and "W_2" are coordinately affected by the gluc-2 mutation. This conclusion was based on the fact that "Y" was present in the conidial washes of all of the esculin-iron positive strains, and it was accompanied by "W_1" and/or "W_2". Neither "W_1" nor "W_2" was found in the absence of "Y". This is what one would expect if "Y" were regulated by gluc-2, but "W_1" and/or "W_2" were independent of that regulation.

The data in Table 5 summarizes the electrophoretic patterns observed from the first and second generation crosses. Since the only means of detecting "W_1" was by electrophoresis, the number of strains which could be grown, harvested, and tested was necessarily limited. The problem was accentuated by the low germination ratio, which could have affected the phenotypic ratios observed in the F_1 and F_2 generations. The data will be discussed below from several different perspectives:

1. Allelic genes (genes at the same locus which have the same function) could be directly or indirectly responsible for the production of "W_1" and "W_2". If this were true, there should be a one-to-one ratio of "W_1" progeny and "W_2" progeny. The data indicates that "W_1" and "W_2" are not mutually exclusive, therefore, this interpretation is improbable.

2. A structural gene could be dictating the structure of "W_1", while "W_2" could be due to environmental or genic modification of "Y" (or of "X").

If this were the case, we would be concerned only with the presence or absence of "W_1". If we assume that "Y" is present in all of the conidial washes, then the following phenotypes would be observed (Table 7):

TABLE 7

NUMBERS OF RANDOM PROGENY WITH W_1^+ AND W_1^- PHENOTYPES

CROSS	W_1^+	W_1^-
M-1	7	2
M-2	14	2
M-3	16	10
M-5	13	10
M-6	17	5
M-7	21	8

These ratios are nonsensical for the expected 50% W_1^+ : W_1^- ratio in a haploid organism (except in the M-5 cross where a 13:10 ratio is close to a 1:1 ratio).

3. A structural gene could be responsible for the structure of "W_2", while "W_1" could be due to environmental or genic modification of "Y" (or

"X"). If this were true, we could be concerned only about the presence or absence of "W_2", and the following phenotypes could be observed (Table 8):

TABLE 8

NUMBERS OF RANDOM PROGENY WITH W_2^+ AND W_2^- PHENOTYPES

CROSS	W_2^+	W_2^-
M-1	6	3
M-2	6	10
M-3	14	8
M-5	23	0
M-6	16	6
M-7	22	7

Again, the ratios do not resemble the expected 1:1 distribution of phenotypes, and the supposition is not supported by the data.

4. "W_1" and "W_2" could be caused by independently segregating genes. Assuming that "Y" is present in all strains, the following phenotypic ratios could be expected from a $W_1^+W_2^-$ (Class 3 in Figure 1) X $W_1^-W_2^+$ (Class 5) cross: 1 $W_1^+W_2^+$: 1 $W_1^+W_2^-$: 1 $W_1^-W_2^+$: 1 $W_1^-W_2^-$. Numbers of progeny (from each cross) having the different phenotypes are summarized in Table 9.

I would like to propose an explanation for these observations. Suppose a $\underline{W_1^+}$ gene were responsible for the "W_1" band and a $\underline{W_2^-}$ gene were responsible for "W_2". Then the cross, Class 3 (W_1^+ W_2^+) X Class 5 (W_1^- W_2^-) would give the following ratio: 1 W_1^+ W_2^+ : 1 W_1^+ W_2^- : 1 W_1^- W_2^+ : 1 W_1^- W_2^-. If W_1^+ W_2^+ is phenotypically a Class 3

TABLE 9

INDEPENDENT SEGREGATION OF W_1 AND W_2 IN RANDOM PROGENY

CROSS	$W_1^+ W_2^+$	$W_1^+ W_2^-$	$W_1^- W_2^+$	$W_1^- W_2^-$
M-1	4	3	2	0
M-2	4	10	2	0
M-3	8	8	6	4
M-5	13	0	10	0
M-6	11	6	5	0
M-7	14	7	8	0

electrophoretic pattern and $W_1^- W_2^-$ is a Class 5 electrophoretic pattern, it is conceivable that both $W_1^+ W_2^-$ and $W_1^- W_2^+$ could result in a Class 6 pattern. In crosses M-5 and M-6, a 1:2:1 ratio, such as this, is observed. This supposition could also explain the results of M-4, where Class 5 ($W_1^- W_2^-$) X Class 6 ($W_1^+ W_2^+$) resulted in ten of the progeny resembling Class 5 and 13 of them resembling Class 6.

Crosses M-2 and M-3, which are reciprocal first generation crosses, do not fit as neatly into this interpretation. This could be due to the fact that the first generation cross was between an exotic strain (of unknown genotype) and a wild-type strain. If the two strains were only distantly related, the cross might have barely been compatible, germination was low, and seemingly obscure ratios could have resulted from non-viable genotypes.

Although no definite conclusions have been drawn about the genetic basis of "W_1" and "W_2", it is apparent that a new area has been opened up. If germination ratios increase in third and fourth generations (as

parental genotypes become more similar), it may be possible to work with greater numbers of progeny to determine statistically significant genetic data which could prove or disprove one of the suggested mechanisms of genetic action.

SUMMARY

A new β-glucosidase, "W_1", was demonstrated by electrophoretic analysis to be in conidial washes of several exotic strains of Neurospora crassa. The biochemical and genetic characteristics of "W_1" were compared with those of established aryl β-glucosidase and cellobiase in order to study diversity in a species.

Aryl β-glucosidase and "W_1" in the conidial wash of exotic strain of P-212 were separated by three methods: electrophoresis on cellulose polyacetate, where "W_1" was less mobile; Bio-Gel P-200 column fractionation, where "W_1" (the smaller enzyme) was eluted last; and calcium phosphate gel adsorption, where aryl β-glucosidase was adsorbed, and "W_1" was in the supernatant fraction.

The physical properties of "W_1" were determined using a purified preparation of "W_1", which was obtained after treatment of a conidial wash with calcium phosphate gel. The new enzyme was relatively thermostable at 60 C, with a half-life of 16 - 17 minutes. The enzyme had a pH optimum of 5.0 in potassium phosphate and in citrate phosphate buffer. A Michaelis constant of 1.98×10^{-5} Molar p-nitrophenyl β-D glucose was determined for Neurospora "W_1".

Although "W_1" was found in conidial washes of strains only 72 hours old (the first day of conidiation), attempts to induce the production of "W_1" in mycelia of any age were unsuccessful.

The genetic studies showed that "W_1" was passed to succeeding generations in crosses with wild-type strains. The new β-glucosidase and aryl

β-glucosidase, but not cellobiase, appeared to be coordinately regulated by the gluc locus.

Electrophoretic analysis revealed a second new β-glucosidase, "W_2", in conidial washes of many exotic and wild-type strains. Electrophoretically, "W_2" was always closely associated with aryl β-glucosidase, but attempts to separate the two enzymes by other methods were unsuccessful.

From these studies, it is apparent that Neurospora produces at least three extracellular β-glucosidases. All three seem to be regulated by the gluc locus, and at least two of them (aryl β-glucosidase and "W_1") have similar kinetic properties.

BIBLIOGRAPHY

Bates, William K., Stephen C. Hedman, and Dow O. Woodward. 1967. Comparative inductive responses of two β-galactosidases of Neurospora. J. of Bact. 93:1631-1637.

Beck, Reta S. 1969. Personal communication.

Berger, Leland S. and Bruce M. Eberhart. 1961. Extracellular β-transglucosidase activity from conidia. Biochem. Biophys. Res. Commun. 6:62-66.

Cahn, R.D., N.O. Kaplan, L. Levine, and E. Zwilling. 1962. Nature and development of lactic dehydrogenase. Science. 136:962-969.

Colowick, Sidney P. and Nathan O. Kaplan. 1955. p.76 Methods in Enzymology, Vol. 1. Academic Press, Inc. New York

Coston, M. Bruce and W.F. Loomis, Jr. 1969. Isozymes of β-glucosidase in Dictyostelium discoideum. J. of Bact. 100:1208-1217.

Eberhart, B.M. 1961. Exogenous enzymes of Neurospora conidia and mycelia. J. Cell. Comp. Physiol. 58:11-16.

Eberhart, B.M. 1962. Methods for screening β-glucosidaseless mutants of Neurospora. Microb. Genet. Bull. 18:27.

Eberhart, Bruce M. 1968. The biochemical genetics of the β-glucosidases of Neurospora. Research Proposal to the National Science Foundation.

Eberhart, B.M. and R.S. Beck. 1969. Personal communication.

Eberhart, Bruce M. and Reta S. Beck. 1970. Localization of the β-glucosidases in Neurospora crassa. J. of Bact. 101:408-417.

Eberhart, Bruce, David F. Cross, and Lewis R. Chase. 1964. β-glucosidase system of Neurospora crassa. I. β-glucosidase and cellulase activities of mutant and wild-type strains. J. Bact. 87:761-770.

Fleming, Leslie W. and Jacob D. Duerksen. 1967a. Purification and characterization of yeast β-glucosidases. J. of Bacteriol. 93: 135-141.

Fleming, Leslie W. and Jacob D. Duerksen. 1967b. Evidence for multiple molecular forms of yeast β-glucosidase in a hybrid yeast. J. of Bact. 93:142-150.

Gilbert, W. and B. Müller Hill. 1966. Isolation of the lac repressor. Proc. Nat. Acad. Sci. 56:1891-1898.

Gilbert, W. and B. Müller Hill. 1967. The lac operator is DNA. Proc. Nat. Acad. Sci. 58:2415-2421.

Gross, S.R. 1969. Genetic regulatory mechanisms in the fungi. Ann. Rev. Genetics. 3:395-424.

Halpern, Y.S. and H.E. Umbarger. 1959. Evidence for two distinct enzyme systems forming acetolacetate in Aerobacter aerogenes. J. Biol. Chem. 234:3067-3071.

Han, Y.W. and V.R. Srinivasan. 1969. Purification and characterization of β-glucosidase of Alcaligenes faecalis. J. of Bact. 100:1355-1363.

Hanks, David L. and Alfred S. Sussman. 1969a. The relation between growth conidiation and trehalase activity in Neurospora crassa. Amer. J. Bot. 56:1152-1159.

Hanks, David L. and A.S. Sussman. 1969b. Control of trehalase synthesis in Neurospora crassa. Amer. J. Bot. 56:1160-1166.

Hash, J.H. and K.W. King. 1958a. On the nature of the β-glucosidases of Myrothecium verrucaria. J. of Biol. Chem. 232:381-393.

Hash, J.H. and K.W. King. 1958b. Some properties of an aryl β-glucosidase from culture filtrates of Myrothecium verrucaria. J. of Biol. Chem. 232:395-402.

Hill, E.P. and A.S. Sussman. 1963. Purification and properties of trehalase(s) from Neurospora. Arch. Biochem. Biophys. 102:389-396.

Hill, E.P. and A.S. Sussman. 1964. Development of trehalase and invertase activity in Neurospora. J. of Bact. 88:1556-1566.

Horowitz, N.H. 1956. The genetic modification of enzyme structure. Supplement Volume of Cytological Proceedings of the International Genetics Symposia. pp. 623-626.

Horowitz, N.H. and M. Fling. 1953. Genetic determination of tyrosinase thermostability in Neurospora. Genetics. 38:360-374.

Horowitz, N.H., M. Fling, H.L. MacLeod, and N. Sueoka. 1960. Genetic determination and enzymatic induction of tyrosinase in Neurospora. J. Mol. Biol. 2:96-104.

Horowitz, N.H., M. Fling, H.L. MacLeod, and N. Sueoka. 1961. A genetic study of two new structural forms of tyrosinase in Neurospora. Genetics. 46:1016-1024.

Horowitz, N.H. and H. Shen. 1952. Neurospora tyrosinase. J. Biol. Chem. 197:513-520.

Jacob, F. and J. Monod. 1961. Genetic regulatory mechanisms and the synthesis of proteins. J. Mol. Biol. 3:318-356.

Jermyn, M.A. 1952. Fungal cellulases. II. The complexity of enzymes from Aspergillus oryzae that split β-glucosidic linkages and their partial separation. Aust. J. Soc. Res. Ser. B. 5:433-443.

Jermyn, M.A. 1962. Fungal cellulases. Further purification of the β-glucosidase of Stachybotrys atra. Australian J. Biol. Sci. 15:769-786.

Johnson, H.N. 1969. Multiple forms of galactosidase in Neurospora crassa. Ph.D. Thesis. Florida State Univ. (Libr. Congr. Card No. Mic. 69-17, 675) 74p. Univ. Microfilms Ann Arbor, Mich.

Johnson, H.N. and A. Gib DeBusk. 1970a. The β-galactosidase system of Neurospora crassa. I. Purification and properties of the pH 4.2 enzyme. Arch. of Biochem. and Biophys. 138:408-411.

Johnson, H.N. and A. Gib DeBusk. 1970b. The β-galactosidase system of Neurospora crassa. II. Extracellular nature of the pH 4.2 enzyme. Arch. of Biochem. and Biophys. 138:412-417.

Keilin, D. and E.F. Hartree. 1938. In: S.P. Colowick and N.O. Kaplan (ed.), Methods in Enzymology, Vol. 1. Acad. Press, Inc. New York.

Landman, O.E. 1954. Neurospora lactase. II. Enzyme formation in the standard strain. Arch. Biochem. Biophys. 52:93-109.

Landman, O.E. and D.M. Bonner. 1952. Neurospora lactase. I. Properties of lactase preparations from a lactose utilizing and a lactose non-utilizing strain. Arch. Biochem. Biophys. 41:253-265.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Mahadevan, P.R. 1963. Biochemical genetics of aryl β-glucosidase of Neurospora. Ph.D. Thesis. Princeton Univ. 102p.

Mahadevan, P.R. and Bruce M. Eberhart. 1962. A dominant regulatory gene for aryl β-glucosidase in Neurospora crassa. J. of Cell. and Compar. Physiol. 60:281-283.

Mahadevan, P.R. and Bruce Eberhart. 1964a. The β-glucosidase system of Neurospora crassa. II. Purification and characterization of aryl β-glucosidase. Archives of Biochem. and Biophysics. 180: 22-29.

Mahadevan, P.R. and Bruce Eberhart. 1964b. The β-glucosidase system of Neurospora crassa. III. Further studies on an aryl β-glucosidase mutant. Arch. of Biochem. and Biophys. 108:30-35.

Metzenberg, R.L. 1962. A gene affecting the repression of invertase and trehalase in Neurospora. Arch. of Biochem. and Biophys. 96: 468-474.

Metzenberg, R.L. 1963. The purification and properties of invertase of Neurospora. Archives of Biochem. and Biophys. 100:503-511.

Metzenberg, R.L. 1964. Enzymatically active subunits of Neurospora invertase. Biochem. Biophys. Acta. 89:291-302.

Murti, C.R. Krishna and B.A. Stone. 1960. Fractionation of the β-glucosidases from Aspergillus niger. Biochem. J. 78:715-723.

Myers, Martin G. and Bruce Eberhart. 1966. Regulation of cellulase and cellobiase in Neurospora crassa. Biochem. Biophys. Res. Comm. 24:782-785.

Ptashne, M. 1967a. Isolation of the λ phage repressor. Proc. Nat. Acad. Sci. 57:306-313.

Ptashne, M. 1967b. Specific binding of the λ phage repressor to DNA. Nature. 214:232-234.

Trevithick, John R. and R.L. Metzenberg. 1964. The invertase isozyme formed by Neurospora protoplasts. Biochem. and Biophys. Res. Comm. 16:319-325.

Trevithick, John R. and Robert L. Metzenberg. 1966. Molecular sieving by Neurospora cell walls during secretion of invertase isozymes. J. of Bact. 92:1010-1015.

Vogel, H.J. 1956. A convenient growth medium for Neurospora. Microbiol. Genet. Bull. 13:42.

Vogel, H.J. 1964. Distribution of lysine pathways among fungi: evolutionary implications. Amer. Nature. 98:435-446.

Zalokar, M. and Cochrane, V.M. 1956. Diphosphopyridine nucleotidase in the life cycle of Neurospora crassa. Am. J. of Botany. 43(2): 107-110.